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Fructose Phosphates and Their Structural Analogues. Synthesis, Nuclear Magnetic Resonance Studies, and Use in the Exploration of the Active-Site of Phosphofructokinase.

Theodore Alfred william Koerner Jr

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FRUCTOSE PHOSPHATES AND THEIR
STRUCTURAL ANALOGUES. SYNTHESIS,
NUCLEAR MAGNETIC RESONANCE STUDIES, AND
USE IN THE EXPLORATION OF THE ACTIVE-
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The Louisiana State University and
Agricultural and Mechanical College
Ph.D., 1975

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FRUCTOSE PHOSPHATES AND THEIR STRUCTURAL ANALOGUES.
Synthesis, Nuclear Magnetic Resonance Studies, and Use in
the Exploration of the Active-Site of Phosphofructokinase

A DISSERTATION

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

by
Theodore Alfred William Koerner, Jr.
B.S., Louisiana State University, 1970
December, 1975

This dissertation is dedicated to the inspiring example of
three carbohydrate chemists:

E. Fischer (1852-1919),

P. A. Levene (1869-1940),

and

H. O. L. Fischer (1888-1960),

and to my wonderful wife Janice, for her understanding and
unselfish support throughout this difficult task.

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ABSTRACT

Because of its potential use in the preparation of analogues of D-fructose 6-phosphate the dibenzylidene D-fructose (DBF) of Brigl and Gr  ner was re-examined. By use of mass spectroscopy, by IR and NMR spectroscopy, and by use of the shift reagent tris(2,2,6,6-tetramethyl-3,5-heptanedionato) europium, DBF was shown to be 2,3:4,5-di-O-benzylidene-  -D-fructopyranose, apparently in a 6S_4 (D) conformation.

In order to determine their aqueous solution structure (tautomeric and anomeric composition), the Fourier transform ${}^{13}\text{C}$ NMR spectra of D-fructose 6-phosphate (F6P) and D-fructose 1,6-diphosphate (FDP) were obtained. The signal assignments made on the basis of ${}^{13}\text{C}$ chemical shifts and ${}^{13}\text{C}$ - ${}^{31}\text{P}$ spin-spin couplings indicate that the earlier assignments of the C-4 and C-5 resonances of   - and   -fructofuranose in oligosaccharides and D-fructose (Allerhand, A.; Doddrell, D., 1971) should be reversed. Integration of signal intensities yields the following equilibrium composition at 35   and pD=8: F6P,   -anomer 19  2% and   -anomer 81  2%; FDP,   -anomer 23  4% and   -anomer 77  4%. Less than 1.5% keto or hydrated-keto form is present in solutions of either fructose phosphate. Calculation of the thermodynamic constants for anomerization yields the following values for $\Delta G_{\alpha \rightarrow \beta}^{\circ}$ in kcal/mole: F6P, -0.88  .05; FDP,

-0.74 \pm .08. The thermodynamic predominance of the β -anomer in solutions of both substrate and product of phosphofructokinase suggested that this form might be the true substrate of the enzyme. This hypothesis was subsequently tested through the use of substrate analogues.

Closely-isosteric, non-isomerizing analogues of each of the four possible forms of F6P (cyclic α - and β -furanose forms, keto-F6P, and hydrated keto-F6P) were prepared. Two of these analogues, 2,5-anhydro-D-glucitol 6-phosphate (AG6P) and 2,5-anhydro-D-mannitol 1-phosphate (AM1P), are novel and their synthesis and characterization are reported. Only AM1P, the analogue of the β -anomer of F6P, was phosphorylated by phosphofructokinase from rabbit muscle and displayed kinetic constants similar to the natural substrate (K_m 0.41 mM, relative V_{max} 87%). The analogue of the α -anomer of F6P, AG6P, was a competitive inhibitor. The near equality of the kinetic constants (K_m with AM1P of 0.41 mM; K_i with AG6P of 0.34 mM) of these two synthetic analogues indicated that the enzyme had almost equal affinity for them. Apparently, the mere shift from the β -form to the α -form placed the C-1 hydroxymethyl group in a position unfavorable for its phosphorylation and transformed the compound from a substrate to a competitive inhibitor. The analogues of the acyclic keto-form and its hydrate were not bound by phosphofructokinase. Thus, the β -furanose form is the only active substrate form, as

suggested above.

Having determined the configurational specificity at C-2 (anomeric specificity), the configurational specificity at C-3, C-4, and C-5 (epimeric specificity) of the F6P site of phosphofructokinase was investigated by testing the activity of three synthetic ketose phosphates as alternate substrates. These (and their epimerized carbon) included: D-psicose 6-phosphate (C-3), D-tagatose 6-phosphate (C-4), and L-sorbose 6-phosphate (C-5). The Michaelis constants (and relative maximal velocities) were 3.0 mM (45%), 0.054 mM (104%) and 11 mM (15%) respectively. Under the same conditions, D-fructose 6-phosphate had a K_m of 0.043 mM and an arbitrary V_{max} of 100%. The low affinity of the enzyme for D-psicose 6-phosphate indicates that the proper L configuration at C-3 is required for effective binding. Moreover, the D configuration at C-5 seems to be equally important for the proper orientation of the phosphate group of the substrate at the active site of the enzyme. On the other hand, the kinetic constants of D-tagatose 6-phosphate were identical with those of D-fructose 6-phosphate, within experimental error. This indicates that the configuration at C-4 is not essential for activity. In the course of this study, the novel synthesis of D-psicose 6-phosphate as well as an improved procedure for the synthesis of D-tagatose 6-phosphate were developed. All products and intermediates for the synthesis of the above three ketose

phosphates were characterized unequivocally by chemical and physical methods.

CHAPTER I
ELUCIDATION OF THE STRUCTURE OF "DIBENZYLIDENE
FRUCTOSE" BY PHYSICAL METHODS AND USE OF A SHIFT REAGENT

A. Introduction

For several years, we (1-3) have been interested in studying the mechanism of action of the allosteric enzyme phosphofructokinase, which catalyzes the phosphorylation of D-fructose 6-phosphate to D-fructose 1,6-diphosphate. In order to assess the relative importance of various structural features present in this substrate, we have embarked on a program to synthesize structural analogues of D-fructose 6-phosphate from D-fructose and its derivatives. In such an undertaking, selective blocking techniques are essential, and one such reaction is the condensation of D-fructose with benzaldehyde. However, the structure of the product of this reaction has not been unequivocally established.

Brigl and Schinle (4) were the first to treat D-fructose with excess benzaldehyde in the presence of zinc chloride, obtaining a solid product (I), which they identified as "dibenzylidene fructose". Using a series of chemical conversions and a modicum of intuition, Brigl and Widmaier (5) assigned the partial structure 2,3:4,5-di-O-benzylidene-D-fructopyranose to I. Because of the

equivocal nature of the experimental approach used by those workers, and the availability of more sophisticated methods for structural determination, we undertook to reinvestigate this compound. In this chapter is reported the structure of product I as deduced from its spectral properties together with those of its monoacetate, II. Application of a shift reagent, tris[2,2,6,6-tetramethyl-3,5-heptanedionato]europium [Eu(thd)₃], to I permitted assignment of the configuration to the anomeric center.

B. Methods and Materials

Crystalline D-fructose was purchased from Pfanstiehl Laboratories, Inc., and dried two hours in vacuo at 60°C prior to use. Benzaldehyde was reagent grade and obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Acetic anhydride, zinc chloride, and pyridine were reagent grade and the product of the Mallinckrodt Chemical Works (St. Louis, Missouri); the latter was dried over potassium hydroxide prior to use. Zinc chloride was dried by fusing immediately before use.

Mass spectra were recorded on a Varian M-66 spectrometer. Infrared spectra were recorded on a Beckman IR-10 spectrometer using potassium bromide pellets. NMR spectra were recorded on a Varian HA-100 spectrometer at the ambient temperature (35°) of the probe, and are referenced to internal tetramethylsilane (3% v/v).

C. Results

2,3:4,5-Di-O-benzylidene- β -D-fructopyranose (I). —

D-Fructose was treated with benzaldehyde in the presence of zinc chloride at room temperature as described by Brigl and Schinle (4). Recrystallization of the product, three times from carbon tetrachloride and twice from methanol gave pure I, m.p. 160° (identical with the reported (4) value); $[\alpha]_D^{20}$ -25.5° (in chloroform); IR data (ν_{\max} , KBr pellet): 3500 cm^{-1} (broad, OH), 1465 (medium), 1415 (medium), 1075 (broad, multiple, ROR), 760 (sharp, Ph), and 700 (sharp, Ph); proton NMR data (100 MHz, CCl_4): 1.73 δ (1-proton multiplet, OH; exchanges rapidly with D_2O), 3.59-3.75 (2-proton multiplet, H-1 and H-1'), 3.73 (1-proton multiplet, H-6'), 3.91 (1-proton doublet of doublets, H-6, $J_{5,6}$ 2 Hz, $J_{6,6'}$ 13 Hz), 4.14 (1-proton multiplet, H-5), 4.46 (1-proton doublet, H-3, $J_{3,4}$ 2.7 Hz), 4.63 (1-proton doublet of doublets, H-4, $J_{3,4}$ 2.7 Hz, $J_{4,5}$ 8.0 Hz), 5.66 (1-proton singlet, 4,5-benzylidene H), 4.80 (1-proton singlet, 2,3-benzylidene H), and 7.21-7.53 (10 proton multiplet, phenyl protons); (60 MHz, p-dioxane): δ 5.67, 5.83 (1-proton singlets, benzylidene protons of 1,3-dioxolane rings); mass-spectral data (intensity expressed as % of base peak): m/e 356 (15, $\text{M}^{+\cdot}$), 355 (15, $\text{M}^{+\cdot} - \text{H}^{\cdot}$), 325 (25, $\text{M}^{+\cdot} - \cdot\text{CH}_2\text{OH}$), 105, (100, PhCO^+), 77 (60, Ph^+), and 31 (15, $^+\text{CH}_2\text{OH}$).

Elemental analysis — for formula $C_{20}H_{20}O_6$ (356.38),

Calculated: C 67.41, H 5.62

Found: C 67.22, H 5.71

1-0-Acetyl-2,3:4,5-di-0-benzylidene- β -D-fructopyranose

(II). — To a solution of 250 mg (0.70 mmoles) of I in 10 ml of dry pyridine at 0°C was added 1.8 g (1.75 mmoles, 25X excess) of acetic anhydride. After 13 hours at room temperature the reaction mixture was poured in a thin stream into 150 ml of vigorously stirred ice-water. The resulting white precipitate was stirred in the aqueous solution fifteen minutes, collected by suction filtration, washed repeatedly with cold water, and air dried. After drying overnight in vacuo the yield of crude product was 270 mg (97%). Recrystallization from absolute ethanol gave II, m.p. 145° (literature (4) m.p. 145-146°); $[\alpha]_D^{20}$ -42.2° (in chloroform); IR data (ν_{\max} , KBr pellet): 2960 cm^{-1} (medium, CH), 1750 (sharp, C=O), 1460 (medium), 1410 (medium), 1080 (broad, R-O-R), 760 (sharp, Ph), and 710 (sharp, Ph); proton NMR data (100 MHz, CDCl_3): 1.99 δ (3-proton singlet, OAc), 3.92 (1-proton, A portion of ABX, H-6', $J_{5,6}$ 0.6 Hz, $J_{6,6}$ 13.3 Hz), 4.14 (1-proton, B portion of ABX, H-6, $J_{5,6}$ 2.0 Hz, $J_{6,6}$ 13.3 Hz), 4.19 (1-proton, A of AB, H-1', $J_{1,1}$ 11.8 Hz), 4.31 (1-proton multiplet, H-5), 4.48 (1-proton doublet, H-3, $J_{3,4}$ 2.4 Hz),

4.60 (1-proton, B of AB, H-1, $J_{1,1}$, 11.8 Hz), 4.74 (1-proton doublet of doublets, H-4, $J_{3,4}$ 2.4 Hz, $J_{4,5}$ 7.9 Hz), 5.78 (1-proton singlet, benzylidene), 5.90 (1-proton singlet, benzylidene), and 7.33-7.66 (10-proton multiplet, phenyl protons); mass spectral data (intensity expressed as % of base peak): m/e 398 (10, $M^{+\cdot}$), 397 (20, $M^{+\cdot}-H^{\cdot}$), 325 (10, $M^{+\cdot} - \cdot CH_2OAc$), 105 (100, $PhCO^+$), 77 (35, Ph^+), 73 (20, $^+CH_2OAc$), and 43 (55, CH_3CO^+).

Elemental analysis — for formula $C_{22}H_{22}O_7$ (398.42),

Calculated: C 66.33, H 5.53

Found: C 66.49, H 5.41

D. Discussion

1. Infrared and Mass Spectra of I and II

The mass spectra of I and its monoacetate II, exhibit molecular ions at the proper mass number for a monomeric, dibenzylidene adduct of a hexulose. Neglecting stereochemical considerations, one can envision in excess of 50 isomeric structures for the monomeric product I. The infrared spectrum of I which indicates the presence of a free hydroxyl function (near 3500 cm^{-1}) and the absence of a carbonyl group, eliminates a number of these possibilities from further consideration. As expected, the infrared spectrum of II is transparent in the hydroxyl region and contains an ester carbonyl absorption at 1750 cm^{-1} .

The major fragments in the mass spectra of I and II indicate the loss of a chain terminal, oxygenated carbon atom as $-\text{CH}_2\text{OR}$. Thus I must have a tricyclic structure with a free hydroxyl group on either C-1 or C-6, a condition that is met by only seven of the structures originally considered.

2. NMR Spectrum of I and II

Additional evidence for the primary nature of the free hydroxyl group of I is obtained from its NMR spectrum in $\text{DMSO}-d_6$ (Fig. I-1). The hydroxyl resonance of I in this solvent appears downfield (5.25 ppm) as a doublet of doubles, owing to slightly unequal coupling to the diastereotopic methylene protons (6), and disappears slowly due to exchange with deuterons of the solvent. As only C-1 and C-6 are attached to two protons, the location of the free hydroxyl group upon one of these two atoms is certain.

Foster's group (7) has shown that the benzylidene protons of 2-phenyl-1,3-dioxolane ring structures resonate at lower field (typically 5.33-5.76 δ) than the analogous protons of 2-phenyl-1,3-dioxane rings. The benzylidene signals of I are observed at 5.67 and 5.85 δ (Fig. I-2), indicating the presence of two, 5-membered, acetal rings in this compound.

The above findings are consistent with two, isomeric structures only, namely 1,2:3,4-di-O-benzylidene-D-fructo-

Fig. I-1. Proton NMR study to reveal the nature of the free hydroxyl of dibenzylidene fructose (I).

Top: spectrum of I in DCCl_3 at 60 MHz. Due to rapid exchange the free hydroxyl proton is observed as a broad envelop at $\sim 2.3 \delta$.

Middle: spectrum of I in $\text{DMSO}-d_6$ at 60 MHz within hours of sample dissolution. Because proton exchange is slow in this solvent the free hydroxyl proton is observed as a clearly resolved doublet of doublets at 5.25δ . This indicates that the free hydroxyl group of I is primary and near a chiral center.

Bottom: spectrum of I in $\text{DMSO}-d_6$ at 60 MHz after several months at room temperature. The slow but eventual exchange of the proton resonating at 5.25δ confirms that this proton is the free hydroxyl proton.

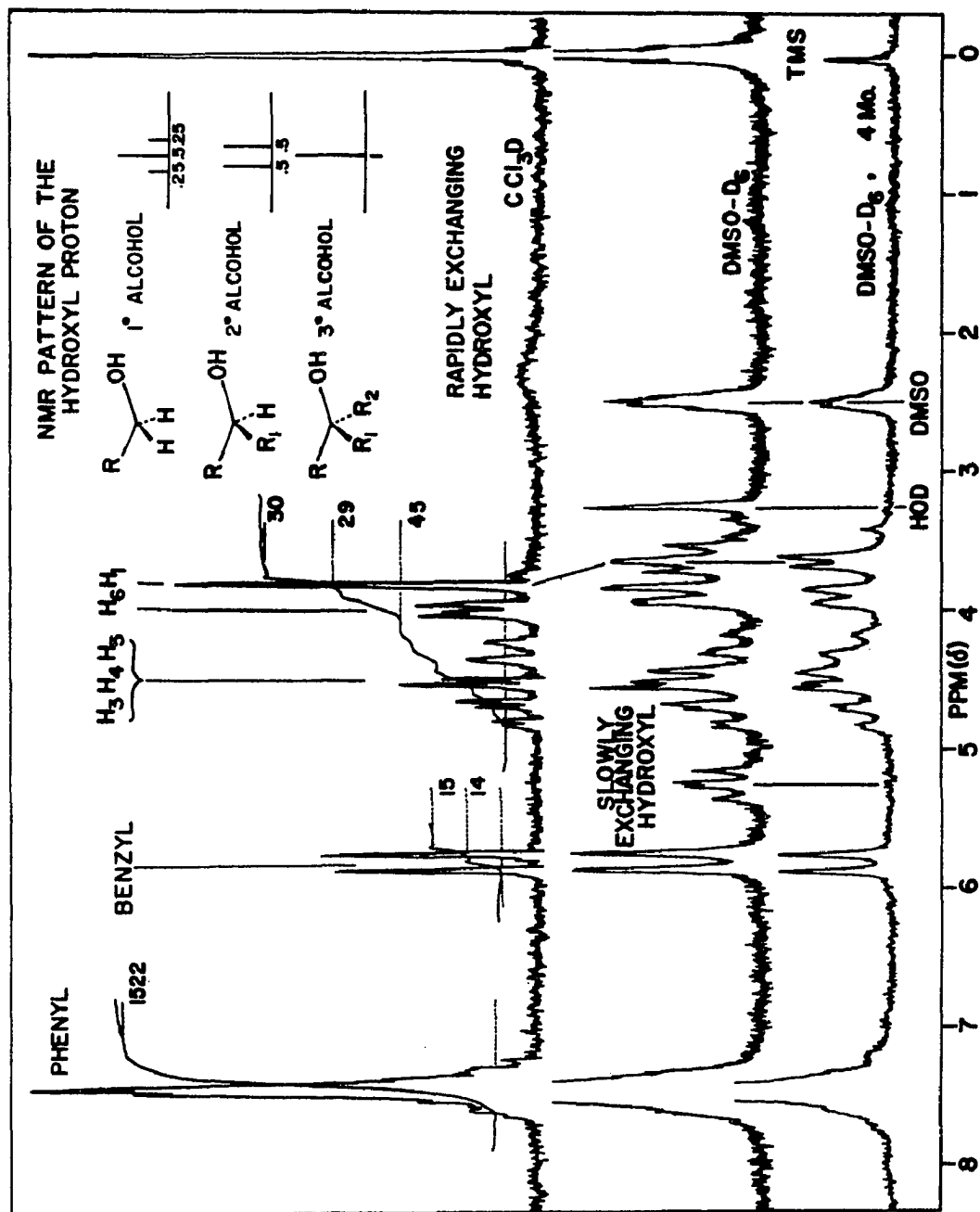


Fig. I-2. Proton NMR spectrum of 10% I in dioxane recorded at 60 MHz. The off-scale dioxane signal has been omitted. To be noted are the benzylidene (benzyl) proton resonances at 5.67 and 5.85 δ . Such downfield resonances are characteristic of benzylidene protons that are contained in 2-phenyl-1,3-dioxolane rings rather than those contained in 2-phenyl-1,3-dioxane rings which resonate further upfield (7).

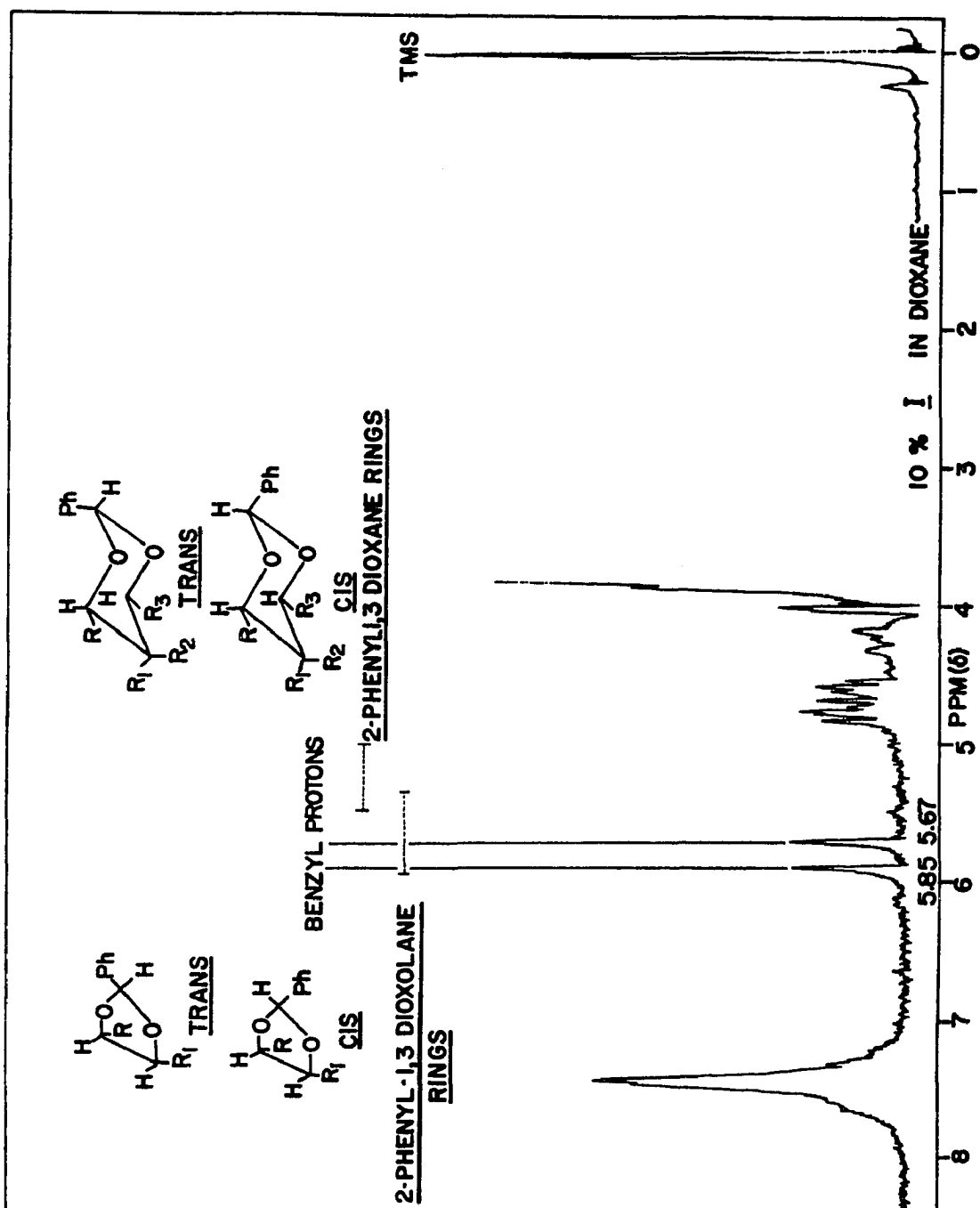
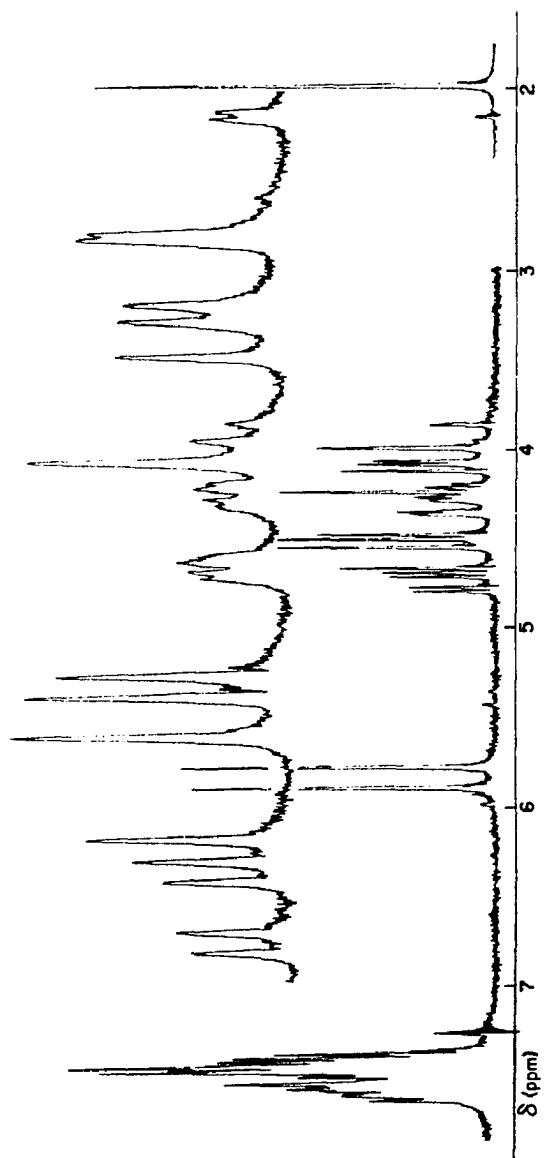


Fig. I-3. Proton NMR spectrum of II recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton.



furanose and 2,3:4,5-di-O-benzylidene-D-fructopyranose. Acetylation of the free hydroxyl group in I is accompanied by a substantial, downfield shift of two signals that are observed as an AB pattern in the 100-MHz NMR spectrum of II (CDCl₃, Fig. I-3); the AB portion of an ABX subspectrum remains at higher field. Whereas H-5 is expected to couple with H-6 and H-6', H-1 and H-1' are insulated from further coupling by the adjacent, quaternary carbon atom (namely C-2). It is thus apparent that the lowfield, AB pattern represents H-1 and H-1', and that the upfield, AB(X) pattern corresponds to H-6 and H-6'. Therefore, the structure 1,2:3,4-di-O-benzylidene-D-fructofuranose can be excluded, and the earlier assignment (5) of I is verified.

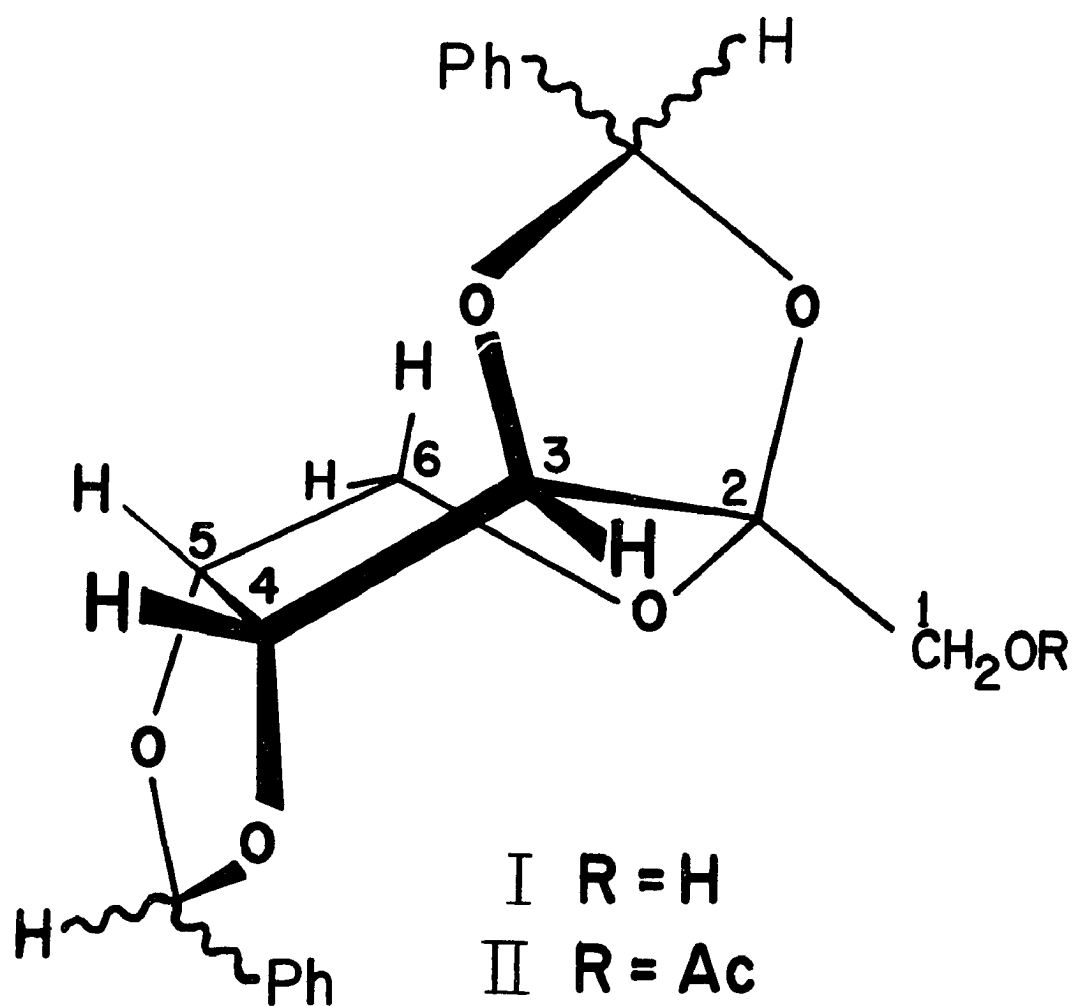
3. Determination of the Anomeric Form of I Through Use of a Shift Reagent

The stereochemistry at every carbon atom, except the anomeric center and the benzylidene groups, is established by the configurational relationships (8) in the parent sugar. Examination of a Dreiding model of the β -anomer of 2,3:4,5-di-O-benzylidene-D-fructopyranose reveals that the separation between O-1 and H-3 is somewhat less than the distance between O-1 and the benzylidene proton. On the other hand, in the α -anomer, these distances are nearly identical. DeMarco et al. (9) have demonstrated an inverse dependence of lanthanide complex-induced NMR signal shifts with such O-H distances. Although I contains six potential

sites for binding of the Lewis acid lanthanide complex (10), tris(2,2,6,6-tetramethyl-3,5-heptanedionato)europium $[\text{Eu}(\text{dpm})_3]$, it has been shown (10-12) that coordination to the hydroxyl group is more efficient than to other oxygen-containing functions. Accordingly, a solution of I in carbon tetrachloride was treated with successively increasing amounts of a saturated solution of $\text{Eu}(\text{dpm})_3$ in the same solvent, and the NMR spectrum was determined at each concentration of the shift reagent. A two-proton signal corresponding to H-1 and H-1' moved downfield at the fastest rate, followed by the H-3 doublet (2.5 and 1.4 times the rate of shift observed for the benzylidene proton of the 2,3-acetal ring, respectively). This observation indicates that H-3 is closer to the paramagnetic center than the 2,3 benzylidene proton, and permits the assignment of the β -D configuration to I.

Taken together, the data obtained from the infrared, mass, and NMR spectra establish unequivocally that I is 2,3:4,5-di-O-benzylidene- β -D-fructopyranose (Fig. I-4). The skew conformation ${}^6\text{S}_4$ illustrated for I (and II) is that adopted by the Dreiding model; in this conformation, the dihedral angles between H-3 and H-4 is $\sim 60^\circ$, and that between H-4 and H-5 is $< 20^\circ$, consistent with the small (2 Hz) and large (8 Hz) values (see RESULTS section) of $J_{3,4}$ and $J_{4,5}$, respectively. The ${}^6\text{S}_4$ conformation has also been determined, by proton NMR spectroscopy, to be the favored conformation of analogous acetal derivatives of aldoses

Fig. I-4. Structures of I and II.



(13) and ketoses (14) having 1,2:3,4-di-O-alkylidene- β -D-arabino stereochemistry (15).

4. Epilogue

The work reported in this chapter ultimately never provided a route to the synthesis of the desired structural analogues of D-fructose 6-phosphate. Subsequent studies revealed that 2,3:4,5-di-O-isopropylidene- β -D-fructopyranose was more easily prepared and served as a better precursor for C-1 derivatization of D-fructose. However, the experience gained by the author in execution of the presently reported work served as an excellent introduction to the implementation of modern analytical techniques for structural studies of carbohydrate derivatives. The laboratory and writing skills mastered in the course of the preparation of this modest work served the author well in the succeeding more fruitful research efforts. During this fledgling undertaking the author relied heavily on his major professor Dr. E. S. Younathan and Dr. J. D. Wander. Both emphasized the importance of thorough and up-to-date laboratory analysis, clear and effective writing, and interdisciplinary collaboration. For this experience the author is deeply appreciative.

In a more condensed form the results presented in this chapter were published in 1972 in the journal Carbohydrate Research under the following reference:

Koerner, T. A. W., Jr., Younathan, E. S., and Wander,
J. D. (1972) Carbohyd. Res. 21, 455.

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CHAPTER II

TAUTOMERIC AND ANOMERIC EQUILIBRIA OF D-FRUCTOSE PHOSPHATES.

DETERMINATION OF EQUILIBRIUM COMPOSITION VIA

¹³C NUCLEAR MAGNETIC RESONANCE.

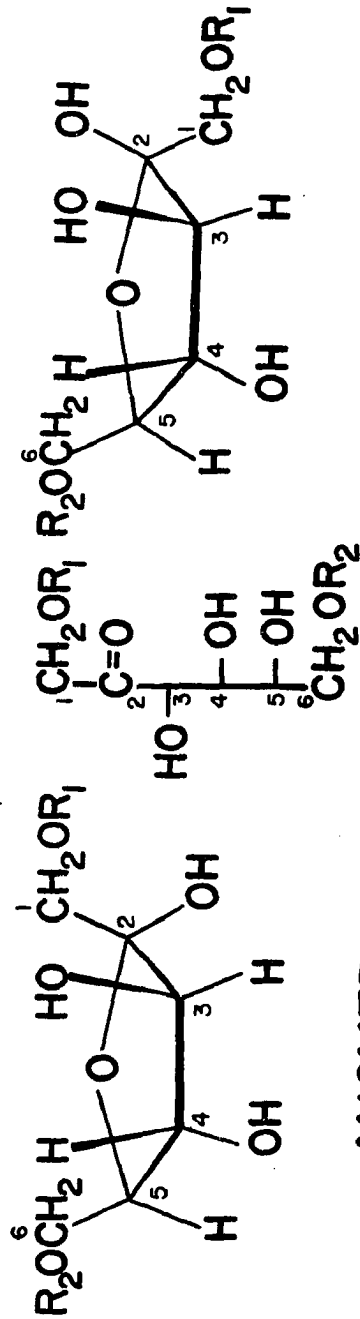
CALCULATION OF KINETIC AND THERMODYNAMIC CONSTANTS

A. Introduction

The phosphate accepting substrate and corresponding product of the key glycolytic enzyme phosphofructokinase are D-fructose 6-phosphate (F6P) and D-fructose 1,6-di-phosphate (FDP) respectively. Three tautomeric forms are possible for each of these reducing sugar phosphates in solution. These include an α -anomer, a β -anomer and a keto form (Fig. II-1). A hydrated keto form (gem diol) can also be envisioned. Since the affinity of phosphofructokinase toward F6P and FDP will probably vary according to their isomeric form, we deemed it meaningful to determine the tautomeric composition of these two sugar phosphates in solution.

Previous investigators have sought to determine the composition of F6P and FDP in solution through ultraviolet (1-4), optical rotatory dispersion (1, 3), circular dichroic (1, 2), infrared (2, 4), proton NMR (4), ³¹P NMR (4, 5), and continuous wave ¹³C NMR (CMR) spectroscopy (4, 6). None of these approaches has been conclusive in

Fig. II-1. Structures of the tautomeric and anomeric forms of D-fructofuranose (FF), D-fructose 6-phosphate (F6P), and D-fructose 1,6-diphosphate (FDP).



α -ANOMER

β -ANOMER

KETO FORM

FF : R_1 & $R_2 = H$
EGP : $R_1 = H$ & $R_2 = PO_3^{-2}$
FDP : R_1 & $R_2 = PO_3^{-2}$

assessing quantitatively the contribution of all tautomeric forms; consequently, a considerable controversy has arisen (2, 5). With the recent development of the sensitive and rapid pulse Fourier transform technique (7), CMR has emerged as a powerful tool in the determination of the tautomeric composition of reducing sugars in solution (8-13). In an attempt to resolve the existing controversy and in continuation of our studies on the molecular basis of the action of phosphofructokinase (14-17) we have applied the Fourier transform CMR method to the determination of the tautomeric composition of F6P and FDP in solution.

B. Materials and Methods

Highest quality D-fructose 6-phosphate, disodium salt, and D-fructose 1,6-diphosphate, tetrasodium salt, were purchased from Sigma Chemical Company. These salts were dissolved in D₂O to form 0.70 M solutions which were submitted to CMR analysis. The pD of F6P and FDP solutions were 8.6 and 7.9 respectively. The CMR spectra were obtained on a Varian XL-100 NMR Spectrometer operating at 23.5 KG for ¹³C acquisition at 25.2 Hz and simultaneous proton noise decoupling at 100 MHz. The utilization of this technique eliminates all ¹³C-¹H spin-spin splitting but retains the coupling patterns caused by ¹³C-³¹P interactions. For ultimate sensitivity enhancement, the spectrometer was operated in Fourier transform mode. Data

were accumulated in a Varian 620i computer employing 2000 Hz sweep width in 4000 points (resolution 0.5 Hz). Data acquisition time and pulse width were 2 sec and 60 μ sec, respectively. Chemical shifts (δ) in parts per million (ppm) were determined relative to internal dioxane and converted to the tetramethylsilane (TMS) scale using $\delta_{\text{C}}^{\text{dioxane}} = +67.40$ ppm. Integrations were obtained electronically after the samples had reached equilibrium (16 hr.).

C. Results and Discussion

1. Tautomeric Composition

Inspection of Fig. II-1 reveals that the best NMR probe of the tautomeric composition of F6P and FDP is the C-2 carbon. In the different electronic environments of the carbonyl, gem diol, R- and S-hemiketal functional groups (keto form, hydrated keto form, α - and β -anomer respectively), the C-2 of F6P or FDP would appear as four separate and distinct resonances in the range 220-90 δ (TMS) (18-21). In fact, the Fourier transform CMR spectrum of each fructose phosphate contains only two resonances in this region. These resonances are readily assigned to the C-2 of the α - and β -anomers (Fig. II-2) by comparison with the previously reported (11) chemical shifts of the C-2 resonances of α - and β -fructofuranose (Table II-1).

The fact that the C-2 resonances of only the α - and β -anomers are detected in the spectra of F6P and FDP

indicates that the keto form or its hydrate are present in concentrations less than the lower limit of sensitivity of the CMR analytical method (0.01 M). This is equivalent to less than 1.5% when calculated on the basis of the total fructose phosphate concentration present (0.70 M). Absence of appreciable keto form is further substantiated by our observation (22) that the proton NMR spectra of both fructose phosphates at 300 MHz reveal no detectable proton exchange after 16 hr. of sample dissolution in basic D₂O. This indicates that labile protons on carbon atoms adjacent to a carbonyl group (characteristic of the keto form) are not present in solutions of F6P and FDP. Thus it is clear that these fructose phosphates in solution do not exist to an appreciable extent in the keto or hydrated keto form as claimed by Avigad et al. (1) and McGilvery (3).

2. ¹³C Assignments

Having established that our CMR spectra contain resonances due only to α- and β-anomers, specific assignments (Table II-1 and Fig. II-2) can be made for F6P and FDP through comparison with the previously assigned resonances of the α- and β-anomers of D-fructofuranose (11). Such a comparison is justified since phosphorylation of a hydroxymethylene carbon deshields it by only a few ppm (23-25). Theoretically, six resonances for each anomer or twelve total ¹³C resonances for each fructose phosphate should be observed. However, resonances of the α-anomer

Fig. II-2. Natural abundance proton-decoupled CMR spectra obtained in D_2O at $35^\circ C$. A, 0.70 M D-fructose 6-phosphate, disodium salt (F6P), $pD = 8.6$, immediately after dissolution (fresh) and B, at equilibrium (16 hr.) with higher amplification (amp.); and C, 0.70 M D-fructose 1,6-diphosphate, tetrasodium salt (FDP), $pD = 7.9$, at equilibrium (16 hr.). The three spectra from top to bottom are the result of 1.7×10^3 , 19.4×10^3 and 13.6×10^3 accumulations. A slight time dependence is observed in the spectrum of F6P, there being 5% more β -anomer present at equilibrium than immediately after dissolution.

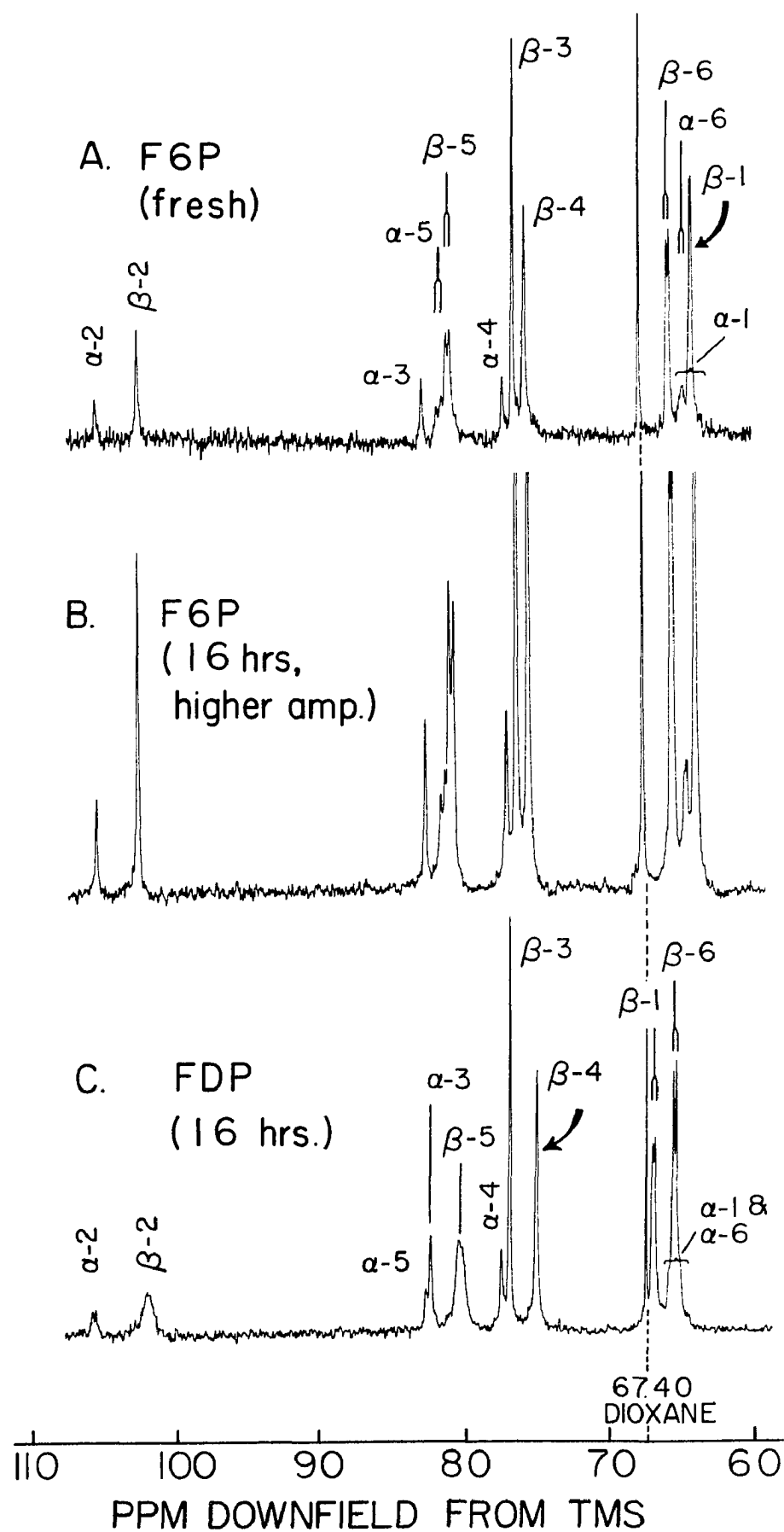


TABLE II-1

^{13}C Chemical Shifts and ^{31}P - ^{13}C Coupling Constants of D-Fructofuranose (FF),^a D-Fructose 6-phosphate (F6P)^b and D-Fructose 1,6-diphosphate (FDP)^b.

| Compound and Tautomeric form | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---------------------------------|--------------------|--------------------|-------|-------|--------------------|--------------------|
| α FF ^a | 62.1 | 105.3 | 83.0 | 77.0 | 82.2 | 62.1 |
| α F6P | c | 105.34 | 82.59 | 76.93 | 81.36 ^e | 64.47 ^f |
| α FDP | d | 105.7 ^g | 82.40 | 77.47 | 82.7 ^g | d |
| β FF ^a | 63.9 | 102.4 | 76.5 | 75.5 | 81.5 | 63.3 |
| β F6P | 63.75 | 102.40 | 76.24 | 75.42 | 80.77 ^h | 65.38 ⁱ |
| β FDP | 66.88 ^j | 102.0 ^g | 76.90 | 75.09 | 80.4 ^g | 65.44 ^k |

a) Shifts for FF reported in (11). To facilitate comparison these shifts, reported previously in the CS_2 scale ($\delta_{\text{C}}^{\text{dioxane}} = -126.20$ ppm), have been converted to the TMS scale using $\delta_{\text{C}}^{\text{dioxane}} = +67.40$ ppm. The α, β C-4 and α, β C-5 shifts of FF have been reassigned (see text).

b) Chemical shifts are expressed in ppm downfield from TMS. The shifts and coupling constants are estimated to be accurate to ± 1 Hz and ± 0.04 ppm except where noted.

c) Buried in F6P β -1 resonance.

d) Buried in FDP β -6 resonance.

e) Doublet, $^3J_{^{31}\text{P}^{13}\text{C}_5} = 8.1$ Hz.

f) Doublet, $^2J_{^{31}\text{P}^{13}\text{C}_6} = 2.8$ Hz.

g) Broadened, coupling is undeterminable, accuracy ± 0.1 ppm.

h) Doublet, $^3J_{^{31}\text{P}^{13}\text{C}_5} = 7.3$ Hz.

i) Doublet, $^2J_{^{31}\text{P}^{13}\text{C}_6} = 4.2$ Hz.

j) Doublet, $^2J_{^{31}\text{P}^{13}\text{C}_1} = 4.0$ Hz.

k) Doublet, $^2J_{^{31}\text{P}^{13}\text{C}_6} = 4.5$ Hz.

C-1 of F6P and FDP and the α -anomer C-6 of FDP are not apparent due to their overlap with the β -anomer C-1 and C-6 resonances.

The assignments for the C-1 and C-6 methylene resonances of D-fructofuranose in oligosaccharides and D-fructose reported earlier (10, 11) are confirmed by our observation of a $^{31}\text{P}-^{13}\text{C}$ coupling in the corresponding resonances of F6P and FDP (Table II-1). This coupling is analogous to that observed in the spectra of nucleotides (23-25). However, it is apparent to us that the earlier assignments (10, 11) of the methine resonances at 82.2 and 81.5 δ (TMS) to the C-4 of the α - and β -anomers of D-fructofuranose respectively are incorrect. Our data indicate that the corresponding resonances in F6P, namely at 81.36 and 80.77 δ (TMS), exhibit a long range $^{31}\text{P}-^{13}\text{C}$ coupling (Table II-1), similar to that observed in the CMR spectra of nucleotides (23-25). The only methine ^{13}C nucleus in F6P that can experience such a long range ^{31}P coupling is C-5. Therefore, the assignments for C-4 and C-5 reported for D-fructofuranose in oligosaccharides (10) and D-fructose (11) should be reversed. These corrections in assignments are incorporated in Table II-1.

3. Anomeric Composition

After specific assignments have been allocated to the α - and β -anomers of F6P and FDP, the percentage of each tautomer present can be measured through integration.

This is feasible quantitatively since Allerhand et al. (24) have shown that all carbons of saccharides have the same nuclear Overhauser enhancement. Comparison of the intensities of the α and β resonances of each carbon in F6P and FDP yields the following equilibrium composition at 35°C: F6P, α -anomer 19 \pm 2% and β -anomer 81 \pm 2%; FDP, α -anomer 23 \pm 4% and β -anomer 77 \pm 4%. A greater error is present in the FDP integration due to more overlap and broadening in the spectrum of FDP (Fig. II-2 and Table II-1).

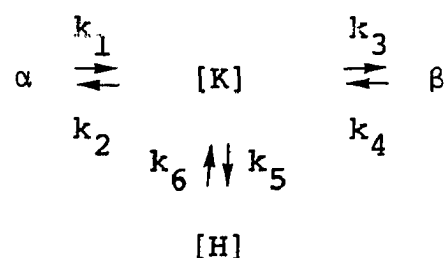
4. Bearing on Previous Investigations

These results are in general agreement with the approximate values for F6P and FDP anomeric composition obtained through continuous wave CMR by Benkovic et al. (6) and through Fourier transform ^{31}P NMR by Gray (5). However, they lend no support to the assumption of the former investigators that F6P exists as much as 5% in the keto form. The use of CMR spectroscopy in the present study seems to provide more accurate data over the ^{31}P NMR approach used by Gray (5) since the data obtained by the latter method are based on the very closely overlapping C-1 and C-6 phosphate ^{31}P resonances of FDP. Moreover, the Fourier transform technique employed in our CMR approach provides certain experimental advantages over the continuous wave technique employed by Benkovic et al. (6). These advantages are: (a) the ability to obtain the entire

CMR spectrum of F6P and FDP rather than the anomeric carbon region only, (b) a greater signal-to-noise ratio and consequently a better integration of resonance intensities, and (c) the avoidance of extensive decomposition of samples due to shorter spectrum acquisition time.

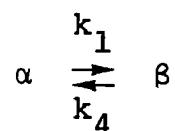
5. Calculation of the Thermodynamic Constants for Anomerization of F6P and FDP

The tautomeric and anomeric equilibria of either fructose phosphate can be completely described by the following four component scheme:



where α , β , K , and H represent the concentrations of the α -anomer, β -anomer, keto-form, and hydrated keto-form, respectively, and k_1 through k_6 are six first-order rate constants. Though similar four-component isomerizations have been subjected to rigorous mathematical analysis and computer solution (26), such an approach is not necessary for F6P and FDP since analysis of their isomerization is readily simplified: Since it was determined above that the concentrations of the keto-form and hydrated keto-form are undetectable the rate constants k_2 , k_3 , k_5 , and k_6

should be much greater than k_1 and k_4 . The above four-component scheme can then be closely approximated by the following two-component scheme:



Such simplification by neglecting forms present in small proportion is with substantial precedence (27).

For this simplified (anomerization) scheme the analytical data for the anomeric composition (supra vide) can be used to calculate K'_{eq} , the apparent equilibrium constant for the $\alpha \rightarrow \beta$ anomerization, using the formula:

$$K'_{eq_{\alpha \rightarrow \beta}} = \frac{\beta}{\alpha} .$$

For D-fructose 6-phosphate at pD 8.6 and 35°C,

$$K'_{eq_{\alpha \rightarrow \beta} \text{ F6P}} = \frac{81 \pm 2}{19 \pm 2} = 4.26 \pm .35 .$$

For D-fructose 1,6-diphosphate at pD 7.9 and 35°C,

$$K'_{eq_{\alpha \rightarrow \beta} \text{ FDP}} = \frac{77 \pm 4}{23 \pm 4} = 3.35 \pm .42 .$$

These calculated values for the apparent equilibrium constants can then be used to calculate the standard Gibb's free-energy change (ΔG°) for the anomerization process at 35°C.

For D-fructose 6-phosphate at pD 8.6,

$$\begin{aligned}\Delta G_{\alpha \rightarrow \beta}^{\circ} \text{ F6P} &= -RT \ln K'_{eq_{\alpha \rightarrow \beta} \text{ F6P}} \\ &= -(1.98 \times 10^{-3} \text{ kcal/}^\circ\text{K} \cdot \text{mole}) (308^\circ\text{K}) \ln(4.26 \pm .35) \\ &= -0.88 \pm .05 \text{ kcal/mole} .\end{aligned}$$

For D-fructose 1,6-diphosphate at pD 7.9,

$$\begin{aligned}\Delta G_{\alpha \rightarrow \beta}^{\circ} \text{ FDP} &= -RT \ln K'_{eq_{\alpha \rightarrow \beta} \text{ FDP}} \\ &= -(1.98 \times 10^{-3} \text{ kcal/}^\circ\text{K} \cdot \text{mole}) (308^\circ\text{K}) \ln(3.35 \pm .42) \\ &= -0.74 \pm .08 \text{ kcal/mole} .\end{aligned}$$

6. Approximation of the Kinetic Constants for Anomerization of F6P and FDP

Recently Wurster and Hess (28, 29) have measured k_1 , the rate constant of the spontaneous anomerization of $\alpha \rightarrow \beta$ F6P and $\alpha \rightarrow \beta$ FDP at 25°C and pH 7.6. If we assume that k_1 and k_4 will both be decreased by a similar factor due to the temperature difference and that the effect of the

difference between the pH of the Wurster and Hess experiment and the pD of our experiments is small (30), then k_4 , the rate constants of the spontaneous anomerization of $\beta \rightarrow \alpha$ -anomer at 25°C, can be approximated for each fructose phosphate using the apparent equilibrium constants calculated above and the following relationship:

$$K'_{eq} = \frac{\text{forward rate}}{\text{reverse rate}} = \frac{k_1}{k_4} .$$

For D-fructose 6-phosphate, $k_1 = 1.6/\text{sec}$ (28) and $K'_{eq_{\alpha \rightarrow \beta}} = 4.26 \pm .35$; therefore at 25°C,

$$k_4 \cong \frac{k_1}{K'_{eq_{\alpha \rightarrow \beta}}} \cong \frac{1.6/\text{sec}}{4.3 \pm .4} \cong 0.37 \pm .03 / \text{sec} .$$

For D-fructose 1,6-diphosphate, $k_1 = 0.55/\text{sec}$ (29) and $K'_{eq_{\alpha \rightarrow \beta}} = 3.35 \pm .42$; therefore at 25°C,

$$k_4 \cong \frac{k_1}{K'_{eq_{\alpha \rightarrow \beta}}} \cong \frac{0.55/\text{sec}}{3.4 \pm .4} \cong 0.16 \pm .02 / \text{sec} .$$

7. Physiological Implications

The thermodynamic predominance of the β -anomer in solutions of both substrate and product of phosphofructokinase suggests that this tautomer might be the form of fructose 6-phosphate involved in the enzyme-substrate complex. We have obtained further experimental evidence in support of this contention from a study on the structural

specificity of rabbit muscle phosphofructokinase with respect to the phosphate acceptor substrate. The latter findings will be reported in subsequent chapters.

Most of the material presented in this chapter was published in 1973 in the journal Biochemical and Biophysical Research Communications under the following reference:

Koerner, T. A. W., Jr., Cary, L. W., Bhacca, N. S.,
and Younathan, E. S. (1973) Biochem. Biophys.
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CHAPTER III
AN EXPLORATION OF THE FRUCTOSE 6-PHOSPHATE SITE
OF PHOSPHOFRUCTOKINASE.
TAUTOMERIC AND ANOMERIC SPECIFICITY

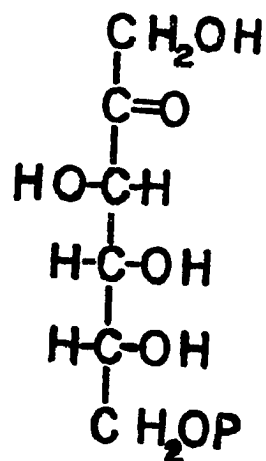
A. Introduction

Detailed knowledge of substrate specificity is important for the delineation of a stereochemical model of the active-site and mechanism of action of an enzyme and is indispensable in the rational design of active-site-directed reagents. Despite its well documented regulatory role in glycolysis (1, 2), little is known of the substrate specificity of phosphofructokinase (EC 2.7.1.11). This investigation is the first in a series that will explore the fructose 6-phosphate site of phosphofructokinase with analogues of its substrate and is a continuation of our studies on the molecular basis of action of this enzyme (3-9).

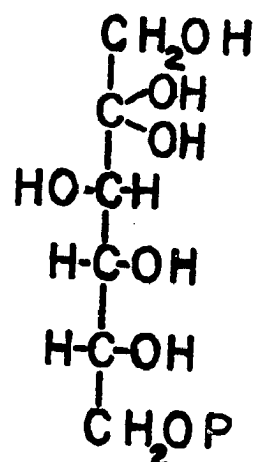
D-Fructose 6-P^{*} is the phosphoryl-acceptor substrate of phosphofructokinase. As with most reducing sugars, it has the potential to isomerize in solution, both constitutionally and configurationally (Fig. III-1). Two readily

* In this and subsequent chapters the abbreviation "P" is used to symbolize the phosphate or $-\text{PO}_3^{-2}$ functional group.

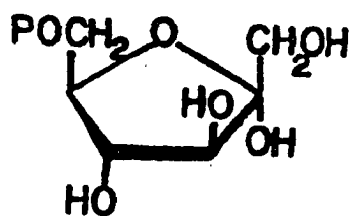
Fig. III-1. Structures of the possible tautomeric and anomeric forms of D-fructose 6-phosphate (I) in solution. They are: I α , α -D-fructofuranose 6-P; I β , β -D-fructofuranose 6-P; I k , Keto-D-fructose 6-P; and I h , hydrated Keto-D-fructose 6-P.



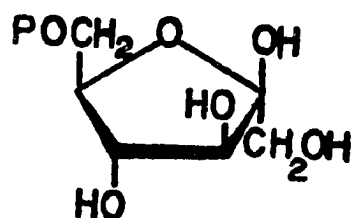
KETO-D-FRUCTOSE 6-P
I_k



HYDRATED KETO FORM
I_h



α-D-FRUCTOFURANOSE 6-P
I_α



β-D-FRUCTOFURANOSE 6-P
I_β

interconvertible ring-chain tautomers¹ (constitutional isomers) are possible. These include an acyclic keto form (I_k) and a cyclic furanose form. Two anomers (C-2 configurational isomers) are possible for the furanose form. These include an α -anomer (I _{α}) and a β -anomer (I _{β}). A small portion of the keto form would be expected to exist as a hydrate (I_h). Thus, there are potentially four different chemical species available to phosphofructokinase in solutions of its phosphoryl acceptor substrate. This possibility poses the question of whether the enzyme can act preferentially on one or more of these different forms of fructose 6-P during binding and phosphorylation.

Recently, we (Chapter II, and Ref. 7) have examined solutions of fructose 6-P and fructose 1,6-P₂ with ¹³C-NMR spectroscopy and have shown that the β -anomer is the predominant form of both compounds at the pH and temperature commonly used for the assay of phosphofructokinase. The actual equilibrium composition of both fructose phosphates was approximately 80% of the β -anomer and 20% of the

¹ The term ring-chain tautomerism is used in this paper to describe the facile cyclic-acyclic constitutional isomerism of reducing sugars. No other term is available to differentiate this type of isomerism from the configurational isomerism (anomerism) of a particular cyclic form. A good discussion of tautomerism and its different forms is presented in reference no. (68).

α -anomer with no detectable amount of the keto or hydrated keto form. The thermodynamic predominance of the β -anomer in solutions of both substrate and product of phosphofructokinase has led us (7, 8) to propose that it is this form that is phosphorylated by the enzyme. On the other hand, previous investigators have emphasized the importance of the α -anomer of fructose 6-P. Schray et al. (10) have maintained that the α -anomer is the only form utilized by rabbit muscle phosphofructokinase. More recently, Benkovic et al. (11) reported that this enzyme probably phosphorylates both the α - and β -anomers of fructose 6-P.

The aim of this study was to investigate the tautomeric and anomeric specificity of the fructose 6-P site of phosphofructokinase. We have probed this site with isosteric, nonisomerizing analogues of each of the four forms of fructose 6-P. Two of these analogues are novel and their synthesis and characterization are reported.

B. Materials and Methods

1. Commercial Materials

Yeast hexokinase, rabbit muscle lactate dehydrogenase, pyruvate kinase, NADH, ATP, D-glucosamine hydrochloride, D-mannitol, dihydroxyacetone, D-fructose 6-P, phosphoenolpyruvate, D-mannitol 1-P (IV), D-glucitol 6-P (V), and D-xylulose 5-P (VI) were purchased from Sigma Chemical Co. Elemental analysis and paper chromatography of the last two phosphates showed them to be 96% and 90% pure,

respectively. Benzoyl chloride, acetic anhydride and diphenyl phosphorochloridate were products of Aldrich Chemical Co. DEAE-Sephadex A-25 was obtained from Pharmacia Fine Chemicals Co.

2. General Synthetic and Analytical Procedures

Samples to be analyzed were dried for 12-15 hr at 60° in vacuo over phosphorus pentoxide and paraffin shavings. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Polyol phosphates, isolated or obtained commercially as barium salts, were converted to the corresponding disodium salts for enzymatic studies and diammonium salts for paper chromatography by treatment with an equivalent amount of the appropriate sulfate and removal of the barium sulfate by centrifugation.

All chromatography was carried out at room temperature ($24 \pm 2^\circ$). Thin layer chromatography (TLC) was performed on glass plates coated with Merck silica gel F-254. Sample migration was detected under UV light or in iodine vapor. Descending paper chromatography was carried out on Whatman No. 1 for polyols using Solvent 1 (n-propanol:ethyl acetate:water, 7:1:2 by volume) and on Whatman No. 40 for polyol phosphate diammonium salts using Solvent 2 (isopropyl ether:88% formic acid, 3:2 by volume). The chromatographs were developed with acid-molybdate spray (12) for phosphates and periodic acid-benzidine spray (13) for polyols. The estimated error in all R_f values is ± 0.01 . Preparative

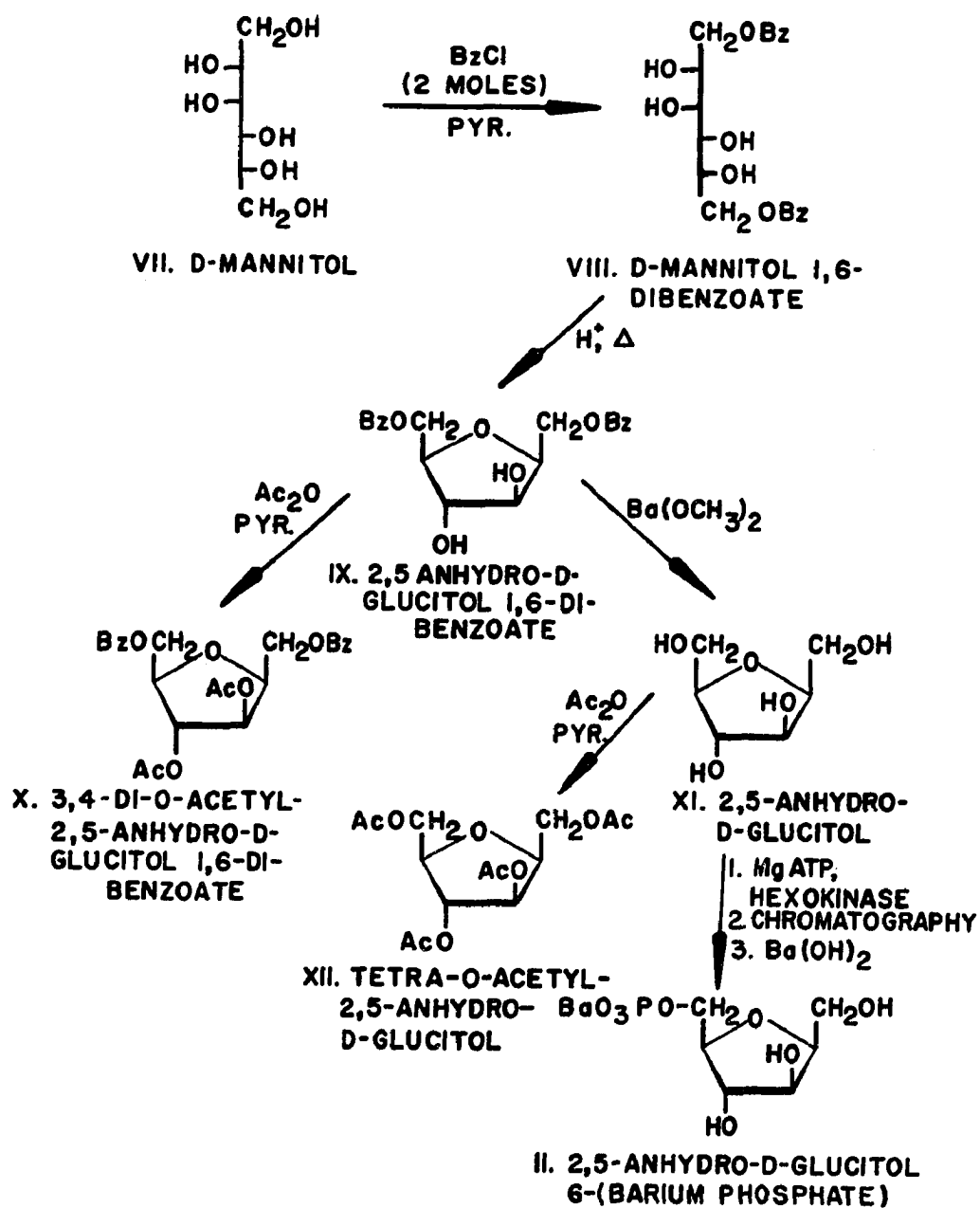
anion exchange chromatography of phosphate esters was similar to that procedure described by Gray (14). The crude polyol phosphate (200-500 mg) was applied in water to a 2x29 cm column of DEAE-Sephadex A-25 (bicarbonate form) and eluted with 900 ml of a linear gradient of ammonium bicarbonate (0.005-0.500 M). Fractions (10 \pm 2 ml) were collected at a flow rate of 30 \pm 10 ml/hr and assayed for total phosphate as described by Bartlett (15) and UV absorption when nucleotides were present.

Melting points were determined by the capillary method and are corrected. Infrared (IR) spectra were obtained with a Perkin-Elmer Model 137 spectrophotometer using a potassium bromide pellet of the sample. Optical rotations were determined with a JASCO J-20 recording spectropolarimeter. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian A-60 and HA-100 NMR-spectrometers.

3. Synthesis and Structural Studies of 2,5-Anhydro-D-glucitol and its Derivatives (Fig. III-2)

Because of its similarity to α -D-fructofuranose 6-P (see DISCUSSION section) we wished to prepare 2,5-anhydro-D-glucitol 6-P. Prerequisite to the synthesis of the 6-phosphate was the preparation of the parent polyol which is reported below. Because of the contradictory physical properties reported for 2,5-anhydro-D-glucitol in the literature (16, 17) we carried out structural studies on this polyol and its derivatives (Fig. III-2) to assure the

Fig. III-2. Synthesis of 2,5-anhydro-D-glucitol (XI) and several derivatives, including 2,5-anhydro-D-glucitol 6-phosphate (II).



validity of the conclusions reached from our enzymatic studies.

2,5-Anhydro-D-glucitol 1,6-dibenzoate (IX). — was the product of benzylation (18) and dehydration (19-21) of D-mannitol (VII). Slow recrystallization from hot benzene afforded fine, white needles, m.p. 139-140°C, $[\alpha]^{20} + 0.75 \pm .05^\circ$ (2.7g per 100 ml of pyridine); literature (20), m.p. 137-138°C, $[\alpha]_D^{20} + 0.93$ (2.7g per 100 ml of pyridine). TLC analysis in ethyl acetate-benzene, 3:2 by volume, showed the product to be homogeneous, R_F 0.29. IR data (λ_{\max} , KBr pellet): 2.93 μ (OH), 3.45 (CH), 5.85 and 5.95 (C=O of benzoate ester), 6.10 (OH), 6.28 (Ph), 6.35, 6.75 (Ph), 6.95 (CH₂), 7.15, 7.30, 7.40, 7.55, 7.65, 7.85 and 7.90 (C-O of benzoate ester), 8.20, 8.50, 8.65, 8.90, 9.00 (C-CH₂-O of benzoylated primary hydroxyl), 9.20 (aliphatic COC), 9.38, 9.50 (secondary hydroxyl of alicyclic 5-membered ring), 9.80, 10.00, 10.20, 10.49, 10.73 (oxolane ring), 10.93, 11.65, 12.18, 12.35, 12.83 (oxolane ring), 13.98 and 14.10 (Ph), 14.60, and 14.80. All peaks not explicitly assigned were characteristic of an aliphatic benzoate ester. These IR assignments as well as those reported hereafter were based on information in Ref. 22 and 23. Proton NMR data (dimethylsulfoxide- d_6 , TMS, 60 MHz): 4.00-4.20 δ (2-proton multiplet, H-3 and H-4), 4.10-4.75 (2-proton multiplet, H-2 and H-5), 4.40-4.55 (4-proton multiplet, H-1, H-1', H-6, and H-6'), 5.37 and 5.42 (two 1-proton doublets, D₂O-exchangeable, C-3 and C-4

hydroxyl protons, $J_{3,\text{OH}} = J_{4,\text{OH}} 4.0\text{Hz}$), and 7.30-8.20 (10 proton multiplet, 2 benzoate phenyls).

Elemental analysis — for formula $\text{C}_{20}\text{H}_{20}\text{O}_7$ (372.38),

Calculated: C 64.51, H 5.41

Found: C 64.33, H 5.46

2,3-Di-O-acetyl-2,5-anhydro-D-glucitol 1,6-dibenzoate
(X). — To 244 mg (0.656 mmole) of thrice recrystallized IX in 10 ml of dry pyridine at 0°C was added with stirring 1.24 ml (13.12 mmoles) of acetic anhydride. After standing 0.25 hr at 0°C and 8.3 hr at room temperature, the clear reaction mixture was poured in a thin stream into 150 ml of vigorously stirred ice water. The resulting emulsion was stirred 10 min, then allowed to stand at room temperature overnight (~12 hr). The upper aqueous layer was then decanted from the precipitated oily product. This oil was taken up in 100 ml of chloroform, washed with 100 ml of 1 N sulfuric acid and 100 ml of saturated sodium bicarbonate solution, dried over anhydrous sodium sulfate, and finally concentrated in vacuo. The resulting oil was dried overnight under high vacuum over paraffin shavings and phosphorus pentoxide to yield 248 mg (83%) of a viscous, yellow oil. Proton NMR data (chloroform-d, TMS, 60 MHz): 2.00 and 2.04 δ (two 3-proton acetate methyls), 4.00-4.40 (2-proton multiplet, H-2 and H-5), 4.45-4.80 (4-proton

multiplet, H-1', H-1, H-6, and H-6'), 5.21 (1-proton doublet of doublets, H-4, $J_{3,4}$ 1.5 Hz, $J_{4,5}$ 3.5 Hz), 5.48 (1-proton multiplet, H-3), and 7.20-8.20 (10-proton multiplet, benzoate phenyls).

Elemental analysis — for formula $C_{24}H_{24}O_9$ (456.45),

Calculated: C 63.15, H 5.30

Found: C 62.92, H 5.61

2,5-Anhydro-D-glucitol (XI). — This polyol was obtained by saponification of 2,5-anhydro-D-glucitol 1,6-dibenzoate (IX). To a solution of 570 mg (1.53 mmoles) of 2,5-anhydro-D-glucitol 1,6-dibenzoate in 25 ml of dry methanol at 0°C was added 1.1 ml (0.55 meq) of freshly prepared 0.5 N barium methoxide. After the reaction mixture had been kept 20 hr at +4°C, barium ions were removed by addition of Dowex 50 cation exchange resin (10 ml, 18 meq, H^+ cycle) and stirring for 3 hr at room temperature. The resin was removed by suction filtration and the filtrate concentrated in vacuo. The resulting yellow syrup was thoroughly extracted ten times with 5 ml portions of diethyl ether to remove the oily contaminant (methyl benzoate). The syrup was taken up in water (25 ml) and treated with Amberlite MB-3 mixed bed exchange resin (10 ml, 18 meq) for 1 hr to remove the last traces of ionic species and the yellow color. Removal of resin by

filtration, concentration of filtrate, and drying at 60°C under high vacuum (8 hr) afforded a clear sirup, which quantitatively crystallized when seeded and scratched; yield 225 mg (90%). Paper chromatography in Solvent 1 showed this material to be homogeneous (R_F 0.50). Its physical constants: m.p. 56°, $[\alpha]_D^{20} +23^\circ$ (2.00 g per 100 ml of water), are in agreement with those of LaMaistre as reported by DeFaye (16), but are in disagreement with those mistakenly assigned to 2,5-anhydro-D-glucitol by Sugihara and Schmidt (17).

Elemental analysis — for formula $C_6H_{12}O_5$ (164.16),

Calculated: C 43.90, H 7.37

Found: C 43.97, H 7.28

Tetra-O-acetyl-2,5-anhydro-D-glucitol (XII). — To 201 mg (1.22 mmoles) of 2,5-anhydro-D-glucitol (XI) in 7.7 ml of dry pyridine at 0°C was added with stirring 4.6 ml (48.8 mmoles, 10 fold excess) of acetic anhydride. After standing 0.5 hr at 0°C and 46 hr at room temperature, the clear reaction mixture was poured in a thin stream into 200 ml of vigorously stirred ice water. The resulting aqueous solution was stirred 0.5 hr then transferred to a separatory funnel and extracted thrice with 70 ml of chloroform. The chloroform extracts were combined, extracted with 100 ml of 1 N sulfuric acid, then 100 ml of

saturated sodium bicarbonate solution, dried over anhydrous sodium sulfate, and finally concentrated in vacuo. The resulting syrup was dried overnight under high vacuum over paraffin shavings to yield 348 mg (86%) of a viscous, yellow syrup that was homogeneous by TLC (R_F 0.55, diethyl ether). Proton NMR data (chloroform- d ; TMS, 60 and 100 MHz): 2.02, 2.04, 2.06, and 2.08 δ (all four, each a 3-proton singlet, 4 acetate methyls), 3.92-4.09 (2-proton multiplet, H-2 and H-5, 4.10-4.42 (4-proton multiplet, H-1, H-1', H-6, and H-6')), 4.98 (1-proton doublet of doublets, H-4, $J_{3,4}$ 1.6 Hz, $J_{4,5}$ 3.1 Hz), and 5.28 (1-proton doublet of doublets, H-3, $J_{3,4}$ 1.6 Hz, $J_{2,3}$ 3.5 Hz). Elemental analysis — for formula $C_{14}H_{20}O_9$ (332.31),

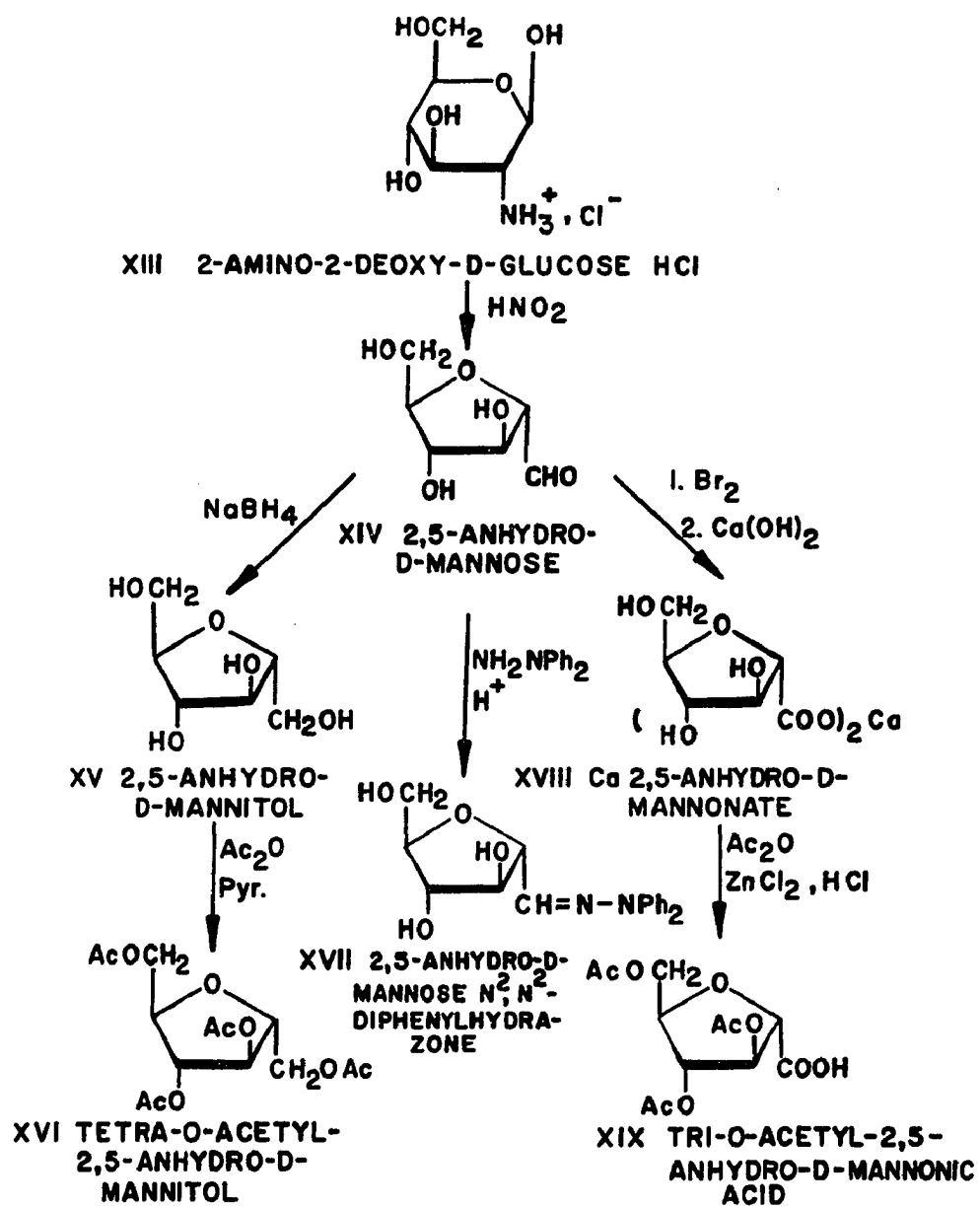
Calculated: C 50.60, H 6.07

Found: C 50.51, H 6.22

4. Synthesis and Structural Studies of 2,5-Anhydro-D-mannitol and its Derivatives (Fig. III-3)

Because of its similarity to β -D-fructofuranose 6-P (see DISCUSSION section) we wished to prepare 2,5-anhydro-D-mannitol 1-P. Prerequisite to the synthesis of the 1-phosphate was the preparation of 2,5-anhydro-D-mannitol (XV), or some derivative of the polyol or its precursor 2,5-anhydro-D-mannose (XIV), that would be suitable for phosphorylation. Initially, a system was sought that was chemically undegenerate with respect to C-1 and C-6. It

Fig. III-3. Synthesis of 2,5-anhydro-D-mannose (XIV),
2,5-anhydro-D-mannitol (XV), and several of
their derivatives.



was hoped that such a system could be appropriately chemically transformed and blocked so that specific monophosphorylation at C-1 could be accomplished. 2,5-anhydro-D-mannose itself was unsuitable because of its instability to temperature, concentration, and base and its inconvenient amorphous nature. Thus several derivatives of XIV were prepared (Fig. III-3) and subjected to chemical modification.

2,5-Anhydro-D-mannose N²,N²-diphenylhydrazone (XVII) was obtained through a literature procedure (24) as a crystalline and stable derivative (vide infra). Repeated attempts to monophosphorylate XVII at C-6 (equivalent to C-1 after C-1 reduction) with diphenyl phosphochloridate; however, were unsuccessful due to the instability of XVII in anhydrous pyridine.

The novel 3,4,6-tri-O-acetyl-2,5-anhydro-D-mannonic acid (XIX) was synthesized (vide infra) using the acetylation procedure of Barker (25). Since Brown and Korytnyk (26) had shown that diborane has an order of functional group reactivity that is greatest for the carboxyl group and least for the ester group, diborane reduction of the triacetyl acid XIX was attempted in the hope of preparing the derivative 3,4,6-tri-O-acetyl 2,5-anhydro-D-mannitol. Such a derivative would have been perfectly blocked for specific monophosphorylation at C-1. However, the C-1 carboxyl group of XIX proved to be remarkable resistant to repeated attempts at diborane reduction with one equivalent

of diborane in dry tetrahydrofuran, yielding only starting material. On the other hand, attempted reduction with one equivalent of diborane in dry tetrahydrofuran-diglyme resulted in partial hydrogenolysis of the acetyl blocking-groups.

The above described difficulty in obtaining 2,5-anhydro-D-mannitol 1-phosphate through specific monophosphorylation of a protected intermediate led us to seek an alternative synthetic strategy. It was decided that a procedure would be sought that would take advantage of the degenerate nature of 2,5-anhydro-D-mannitol (XV). Because of the C_2 -axis of symmetry present in XV phosphorylation at C-6 would be equivalent to reaction at C-1. Thus, all that was needed was to employ a phosphorylating reagent specific for primary hydroxyl groups, then to separate chromatographically the monophosphorylated product from the twice-reacted product. Besides this possible chemical method for obtaining 2,5-anhydro-D-mannitol 1-phosphate, it was discovered that the enzyme yeast hexokinase would phosphorylate 2,5-anhydro-D-mannitol (27). With two good routes to the synthesis of 2,5-anhydro-D-mannitol 1-phosphate thus based on the parent polyol itself, we prepared 2,5-anhydro-D-mannitol (XV). The tetra-O-acetate of XV was prepared as a derivative well suited for proton NMR analysis.

2,5-Anhydro-D-mannose (XIV, "chitose") was prepared by deamination of D-glucosamine hydrochloride (XIII) with

either silver nitrite or nitrous acid according to the published procedures of Fischer and Tiemann (28), Grant (29), and Bera et al. (30). As reported in the literature, the product XIV obtained by all three procedures was a viscous, unstable syrup that could not be induced to crystallize, despite numerous attempts.

2,5-Anhydro-D-mannitol (XV) was prepared from syrupy 2,5-anhydro-D-mannose (XIV) by sodium borohydride reduction according to the procedure of Bera et al. (30). For months the compound could be prepared only as a clear, viscous syrup; however, after obtaining seed crystals and an initial nucleation, the compound thereafter crystallized spontaneously. Seed crystals were the generous gift of Dr. J. W. LaMaistre of Imperial Chemical Industries (America). Recrystallization from hot ethanol yielded large, cuboidal crystals, often in rosettes (Fig. III-4), m.p. 101.5-102.5°C $[\alpha]_D^{20} +58.0 \pm .5^\circ$ (1.37 g per 100 ml of water); literature (30), m.p. 100-101°C, $[\alpha]_D^{20} +58.2^\circ$. Paper chromatography in Solvent 1 showed the product to be homogeneous (R_F 0.47). IR data (λ_{max} , KBr pellet): 3.00 μ (OH), 3.50 (CH), 6.20 (OH), 6.97 (CH₂), 7.20 (CH), 7.70, 8.30 (CH₂), 9.10 (COC), 9.60 (saturated 1° and 2° COH of alicyclic 5-membered ring), 10.27, 10.42, 10.85 (oxolane ring), 11.10, and 12.05 (oxolane ring).

The structure of 2,5-anhydro-D-mannitol was confirmed through nuclear magnetic resonance studies. Assuming that

Fig. III-4. Photograph of a crystalline rosette of 2,5-anhydro-D-mannitol (XV) slowly grown from absolute ethanol (magnification 3X).
Photography courtesy Mr. R. J. Voll and Mr. R. E. Voll.



the C-4 and C-5 D-configurations of the parent compound D-glucosamine hydrochloride (31, 32) are maintained and that a 2,5-anhydro ring is present in XIV and therefore in XV, then demonstration of a C_2 axis of symmetry in XV is proof of the D-manno stereochemistry, since no other stereochemical pattern will impart to XV such high symmetry. Examination of XV at 60 MHz resulted in a poorly resolved proton NMR spectrum; however, at 100 and 300 MHz a well resolved pattern emerged. Manual analysis of the higher frequency spectra according to established techniques (34-38) revealed the 8 non-exchangeable protons of XV to interact as an AA'BB'XX'YY' spin system (more easily visualized if written AB-X-Y-Y'-X'-A'B'), a composite of two equivalent ABX subsystems (H-1, H-1', H-2; and H-6, H-6', H-5) and an XX'YY' subsystem (H-2, H-5, H-3, H-4). The chemical and magnetic equivalence of the two ABX subsystems and presence of an XX'YY' subsystem clearly demonstrates that XV possesses a C_2 axis of symmetry and reveals that this axis passes between H-3 and H-4 and through the ring oxygen so as to divide XV into two equivalent halves (i.e. H-1, H-1', H-2, H-3; and H-6, H-6', H-5, H-4). The complete analysis of the 8-protons of XV at 100 and 300 MHz in D_2O yielded the following data: 3.84 δ (2-proton A portion of ABX, H-1' and H-6', $J_{1,1'} = J_{6,6'} -12.4$ Hz, $J_{1',2} = J_{5,6'} 5.3$ Hz), 3.99 (2-proton B portion of ABX, H-1 and H-6, $J_{1,1'} = J_{6,6'} -12.4$ Hz,

$J_{1,2} = J_{5,6}$ 3.0 Hz), 4.08 (2-proton XX' of $XX'YY'$ additionally coupled to AB and A'B', H-2 and H-5, $J_{1',2'} = J_{5,6}$, 5.3 Hz, $J_{1,2} = J_{5,6}$ 3.0 Hz, $J_{2,3} = J_{4,5}$ 8.5 Hz, $J_{2,4} = J_{3,5}$ -5.7 Hz, $J_{2,5} < 0.1$ Hz), and 4.24 (2-proton YY' of $XX'YY'$, H-3 and H-4, $J_{3,4}$ 3.3 Hz, $J_{2,3} = J_{4,5}$ 8.5 Hz).

The results obtained from the above proton NMR study were further substantiated through examination of the ^{13}C NMR spectrum of XV at 25.2 MHz, operating in the high-resolution pulsed Fourier-transform mode, the ^{13}C spectrum of XV was observed to be only three sharp singlets of equal intensity. These three peaks (and their assignments) expressed as δ (ppm downfield from tetramethylsilane) are: 62.18 (C-1, C-6), 77.41 (C-3, C-4), and 83.20 (C-2, C-5). This demonstration of the chemical equivalence of C-1 and C-6, C-2 and C-5, and C-3 and C-4 clearly indicates the presence of a C_2 axis of symmetry through XV. Thus, examination of both the proton and ^{13}C NMR spectra of XV reveal the symmetry necessary to confirm the 2,5-anhydro-D-manno stereochemistry originally assigned to XV by Bera *et al.* (30) and assigned to XIV by Levene and LaForge (39-41).

Elemental analysis — for formula $\text{C}_6\text{H}_{12}\text{O}_5$ (164.16),

Calculated: C 43.90, H 7.37

Found: C 43.90, H 7.22

Tetra-O-acetyl-2,5-anhydro-D-mannitol (XVI). — To 200 mg (1.22 mmoles) of 2,5-anhydro-D-mannitol in 7.7 ml of dry pyridine at 0°C was added with stirring 4.6 ml (48.8 mmoles, 10 fold excess) of acetic anhydride. After standing 0.5 hr at 0° and 46 hr at room temperature, the clear reaction mixture was poured in a thin stream into 200 ml of vigorously stirred ice water. The resulting aqueous solution was stirred 0.5 hr then transferred to a separatory funnel and extracted thrice with 70 ml portions of chloroform. The chloroform extracts were combined, extracted with 100 ml of 1 N sulfuric acid, then 100 ml of saturated sodium bicarbonate solution, dried over anhydrous sodium sulfate, and finally concentrated in vacuo. The resulting syrup was dried overnight under high vacuum over paraffin to yield 363 mg (90%) of a viscous, yellow syrup that was homogeneous by TLC (R_F 0.50, diethyl ether); $[\alpha]_D^{20} +27.1 \pm 0.5^\circ$ (4.2 g per 100 ml chloroform); literature (42) $[\alpha]_D +27.3^\circ$. Examination of this material (XVI) by proton NMR (chloroform- d , TMS, 60 and 100 MHz) revealed a very degenerate spectrum consistent with the high symmetry (C_2 axis) that is implicit in the assigned structure: 1.89 δ (12-proton singlet, four 3-proton acetate methyls), 4.04 (6-proton singlet, H-1, H-1', H-2, H-5, H-6, and H-6'), and 4.95 (2-proton singlet, H-3 and H-4).

Elemental analysis — for formula $C_{14}H_{20}O_9$ (332.31),

Calculated: C 50.60, H 6.07

Found: C 50.48, H 6.18

2,5-Anhydro-D-mannose N²,N²-diphenylhydrazone (XVII)

was prepared as a crystalline derivative of syrupy 2,5-anhydro-D-mannose (XIV) by treating acidic "chitose" syrup with refluxing ethanolic 1,1-diphenylhydrazine, according to the procedure of Schorigin and Markarowa-Semljanskaja (24). Crystallization from diethyl ether and recrystallization from hot water afforded fine, white needles, m.p. 143-144°C; literature (24) m.p. 144-145°C.

Elemental analysis — for formula C₁₈H₂₀O₄N₂ (328.37),

Calculated: C 65.84, H 6.14, N 8.53

Found: C 65.47, H 6.37, N 8.41

Calcium 2,5-anhydro-D-mannonate (XVIII) was prepared by oxidation of 2,5-anhydro-D-mannose (XIV) with aqueous bromine according to the procedure of Fischer and Tiemann (28). Recrystallization from water by slow evaporation afforded clear, rectangular plates, $[\alpha]_D^{19} +33.0 \pm .5^\circ$ (9.0 g per 100 ml water); literature (28) $[\alpha]_D^{19} +32.8^\circ$ (8.96 g per 100 ml water).

Elemental analysis — for formula (C₆H₁₀O₆·H₂O)₂ Ca (430.38),

Calculated: C 33.49, H 5.15

Found: C 33.15, H 5.26

3,4,6-Tri-O-acetyl-2,5-anhydro-D-mannonic acid (XIX).

— To a dry solution of freshly-fused, pulverized zinc chloride (1.8 g, 13 mmoles) and acetic anhydride (25.0 ml, 258 mmoles) was added with stirring 5.16 g (12.00 mmoles) of calcium 2,5-anhydro-D-mannonate (XVIII). The resulting slurry was chilled to -5° , then saturated with dry hydrogen chloride gas. Gassing and chilling were continued for 15 min after all the calcium salt had dissolved, then the reaction mixture was protected with a calcium chloride drying tube and allowed to stand at room temperature 18 hr. The reaction mixture was chilled to 0° ; whereupon, excess ice was added cautiously to destroy unreacted acetic anhydride. After stirring 1 hr at room temperature the reaction mixture was diluted to 100 ml with water, transferred to a separatory funnel, and extracted eight times with 30 ml portions of chloroform. The chloroform extracts were combined, dried over anhydrous sodium sulfate, and concentrated in vacuo to a yellow syrup. This syrup was co-distilled thrice with 40 ml portions of toluene to remove all traces of acetic acid and dried several days under high vacuum over paraffin shavings to yield 5.9 g (80%) of a viscous, yellow syrup. Proton NMR data (chloroform-d, TMS, 60 MHz); 2.06, 2.09, and 2.13 δ (all three, each a 3-proton singlet, three acetate methyls), 4.29 (2-protons, AB portions of ABX, H-6 and H-6', $J_{5,6} = J_{5,6'}$ 7.0 Hz), 4.40 (1-proton, X portion of ABX additionally coupled to H-4, H-5, $J_{4,5}$ 3.0 Hz, $J_{5,6} = J_{5,6'}$ 7.0 Hz), 4.67 (1-proton

doublet, H-2, $J_{2,3}$ 2.0 Hz), 5.12 (1-proton doublet of doublets, H-4, $J_{3,4}$ 2.0 Hz, $J_{4,5}$ 3.0 Hz), 5.53 (1-proton doublet of doublets, H-3, $J_{2,3}$ 2.0 Hz, $J_{3,4}$ 2.0 Hz), and variably 6-10 (D_2O -exchangeable 1-proton singlet, carboxylic acid proton).

Elemental analysis — for formula $C_{12}H_{16}O_9$ (304.26)

Calculated: C 47.37, H 5.30

Found: C 47.39, H 5.43

5. Assay of Phosphofructokinase Activity

Rabbit skeletal muscle phosphofructokinase was prepared by the method of Ling et al. (43). Enzymatic activity was determined by a coupled assay of the ADP formed by the phosphofructokinase reaction. The assay mixture contained the following reagents at the indicated final concentrations: 1 mM ATP, 4 mM magnesium sulfate, 50 mM potassium chloride, 1 mM dithiothreitol, 0.2 mM EDTA, 0.2 mM phosphoenolpyruvate, 50 mM Tris-Cl buffer (pH 7.8), 0.16 mM NADH, 20 units of pyruvate kinase, 20 units of lactate dehydrogenase and an appropriate amount of phosphofructokinase. The reaction was initiated by the addition of fructose 6-P or its analogues.

The enzymatic activity was followed by monitoring the decrease in absorbance of NADH at 340 nm using a Gilford recording spectrophotometer. An enzyme unit was defined as the amount of phosphofructokinase which catalyzes the

the conversion of 1 μ mole of fructose 6-P to fructose 1,6-P₂ per minute at pH 7.8 and 28°. Protein concentration was determined from the absorbance at 290 nm in 0.1 N sodium hydroxide solution (4). Kinetic data were depicted as a Lineweaver and Burk (44) plot for the substrates and as a Dixon (45) plot for the inhibitor. The kinetic constants were calculated from the best fitting regression lines generated through an iterative least squares analysis of the data. This analysis was programmed in Fortran using a slight modification of the program recommended by Cleland (46) and performed on an IBM 360 computer.

Enzymatic assays were performed by Mr. A. E. Ashour (47) under the supervision of Dr. E. S. Younathan. Computer programming was performed by Mr. R. J. Voll in close association with the author.

C. Results

1. Synthesis of 2,5-Anhydro-D-glucitol 6-phosphate (II)

This analogue was prepared from the parent polyol by ATP dependent enzymatic phosphorylation. In light of its known specificity (48), yeast hexokinase was used to selectively phosphorylate the C-6 hydroxyl of the polyol (Fig. III-2). The composition of the enzymatic reaction mixture was as follows: 2,5-anhydro-D-glucitol (154 mg, 0.94 mmoles), disodium ATP trihydrate (812 mg, 1.34 mmoles) and magnesium dibromide hexahydrate (410 mg, 1.40 mmoles) in 20 ml of water. The pH of the mixture was adjusted to

7.5 with 0.2 N sodium hydroxide solution, and 4.1 ml (1670 units) of a crystalline suspension of the enzyme was added in several increments. The reaction was allowed to proceed at room temperature for 24 hr with gentle stirring, maintaining the pH at 7.5 by dropwise addition of 0.2 N sodium hydroxide solution. The reaction mixture was then chromatographed on a DEAE-Sephadex A-25 column as described under Section B, Part 2. Fractions containing pure 2,5-anhydro-glucitol 6-P were combined, acidified with Dowex 50 (H^+ form) and filtered. The clear filtrate was immediately neutralized to pH 10.3 with saturated barium hydroxide solution and filtered. The filtrate was concentrated to obtain the amorphous barium salt of 2,5-anhydro-glucitol 6-P; yield 120 mg (36%). As its diammonium salt, this material was chromatographically homogeneous (R_F 0.28) on paper in Solvent 2; $[\alpha]_D^{20} +11^\circ$ (2.88 g of the disodium salt per 100 ml of water); IR data (barium salt, λ_{max}): 3.00 μ (OH), 3.45 (CH), 5.80, 6.20 (OH), 7.00 (CH), 7.20 (CH), 8.28, 8.98 (COC), 9.25 (P=O), 10.20 (POC), 10.73, 11.23, and 12.50 (POC).

Elemental analysis — for formula $C_6H_{11}O_8PBa \cdot 2 H_2O$ (415.49),

Calculated: C 17.34, H 3.64, P 7.45

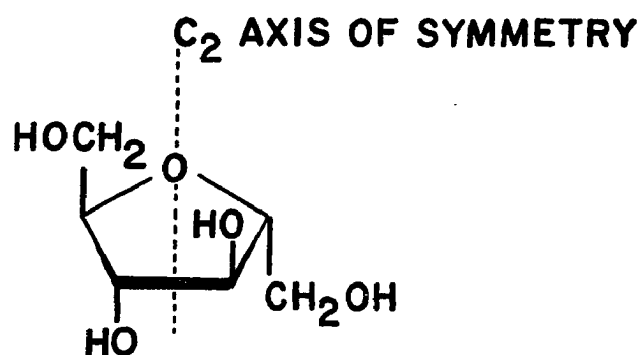
Found: C 17.27, H 3.66, P 7.44

2. Synthesis of 2,5-Anhydro-D-mannitol 1-Phosphate² (III)

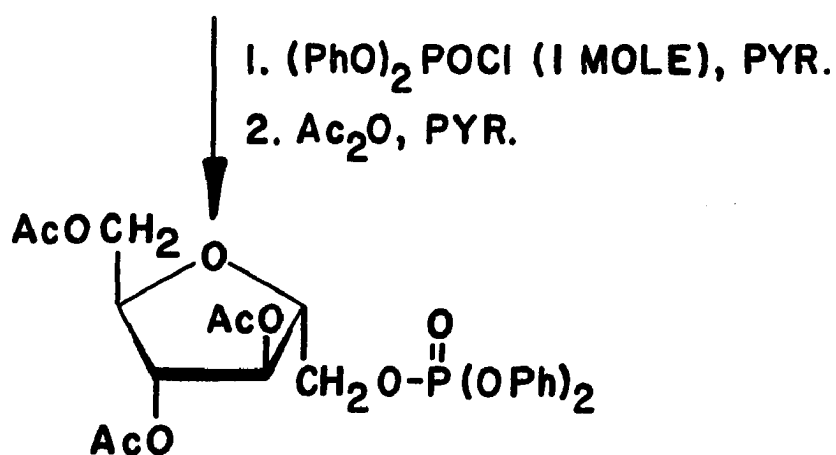
Small quantities of this analogue were prepared enzymatically in a manner identical to that described above for the anhydroglucitol analogue. However, a simple chemical procedure was desired for the synthesis of large quantities of 2,5-anhydro-D-mannitol 1-P. In view of the C_2 axis of symmetry present in 2,5-anhydro-D-mannitol and the predilection of the phosphorylating reagent for primary hydroxyl groups (49) we were able to synthesize this analogue without the preliminary introduction of blocking groups (Fig. III-5). Diphenyl phosphorochloridate (3.28 g, 12.2 mmoles) was added dropwise to a solution of 2,5-anhydro-D-mannitol (2.0 g, 12.2 mmoles) in 40 ml of dry pyridine at -20° . After the sealed reaction mixture had been kept at -20° for 1 hr, at 0° for 24 hr, and at room temperature for 1 hr, 24.0 ml (256 mmoles) of acetic anhydride was added. The resulting solution was allowed to stand at room temperature for 15 hr, then was poured with vigorous stirring into 800 ml of ice water. The resulting emulsion was allowed to separate over 30-40 hr at 4° and the aqueous layer was decanted and discarded. The precipitated oil was taken up in 200 ml of chloroform and

² It is to be noted that the C_2 axis of symmetry through D-mannitol and 2,5-anhydro-D-mannitol makes their 1- and 6-phosphates equivalent. Rules of nomenclature dictate the use of the lower number.

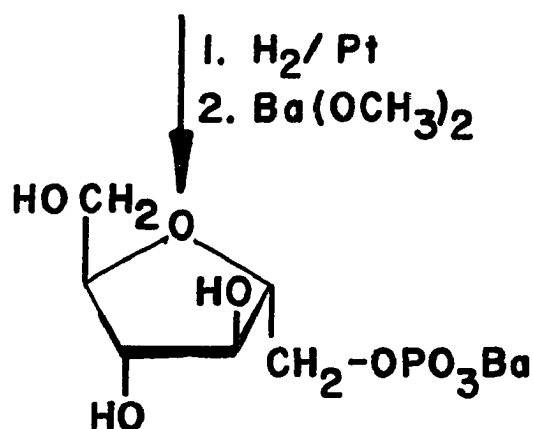
Fig. III-5. Synthesis of 2,5-anhydro-D-mannitol 1-phosphate (III).



XV. 2,5-ANHYDRO-D-MANNITOL



XX. 1-O-DIPHENYLPHOSPHORYL-3,4,6-TRI-O-ACETYL-2,5-ANHYDRO-D-MANNITOL



III. 2,5-ANHYDRO-D-MANNITOL 1-(BARIUM-PHOSPHATE)

washed successively with 100 ml of each of the following: 1 N sulfuric acid, saturated sodium bicarbonate solution and finally water. The chloroform layer was dried over anhydrous sodium sulfate, filtered and concentrated to give 5.2 g (82%) of a clear oil. TLC (diethyl ether) showed that this oil contained a major (75 mole%) component (R_F 0.31) and a minor (25 mole%) component (R_F 0.24). The former was identified as 1-diphenylphosphoryl-3,4,6-tri-O-acetyl-2,5-anhydro-D-mannitol (XX) and the latter as 1,6-bis-(diphenylphosphoryl)-3,4-di-O-acetyl-2,5-anhydro-D-mannitol by comparison with known standards. A solution of this oil (5.2 g) in 100 ml of methanol was hydrogenated over platinum oxide (150 mg) at two atmospheres of pressure. After the uptake of 100.3 mmoles (102%) of hydrogen, the reaction was complete (30 hr). The catalyst was removed by filtration and the filtrate was neutralized with cold 0.2 N barium methoxide solution. After 10 min at 0°, de-acetylation was complete and the methanol solution became a clear gel. Addition of a quarter volume of water resulted in the formation of a white precipitate which was allowed to flocculate at 4° overnight. This precipitate was collected by centrifugation and washed successively with 20 ml of each of the following: anhydrous methanol, 3:1 methanol-ether, 1:3 methanol-ether and anhydrous ether. Drying overnight yielded 2.9 g (58%) of the crude barium salt of 2,5-anhydromannitol 1-P. Paper chromatography of the diammonium salt of this material in Solvent 2

revealed the product (R_F 0.25) to be contaminated with 25 mole% of 2,5-anhydro-D-mannitol 1,6-P₂ (R_F 0.17) and a trace of orthophosphate (R_F 0.45). Repeated attempts to purify the crude barium salt by fractional precipitation with ethanol-water (50) were unsuccessful. Contaminants were removed, however, by DEAE-Sephadex A-25 column chromatography and the barium salt of pure 2,5-anhydro-mannitol 1-P was obtained by a procedure similar to that described above for the anhydroglucitol analogue. The yield was 1.35 g (27% overall). Examination of the diammonium salt of this material by paper chromatography using Solvent 2 showed a single spot (R_F 0.25); $[\alpha]_D^{20} +28^\circ$ (2.88 g of the disodium salt per 100 ml of water); IR data (barium salt, λ_{max}): 3.00 μ (OH), 3.48 (CH), 6.18 (OH), 7.00 (CH), 7.20 (CH), 9.00 (COC), 9.25 (P=O), 10.23 (POC), 10.95, 11.65, and 12.50 (POC).

Elemental analysis — for formula $C_6H_{11}O_8P\text{Ba} \cdot 2 H_2O$ (415.49),

Calculated: C 17.34, H 3.64, P 7.45

Found: C 17.30, H 3.67, P 7.39

3. Effect of Analogues on Phosphofructokinase Activity

2,5-anhydro-D-mannitol 1-phosphate² was found to act as an alternate substrate for phosphofructokinase. That this analogue was a substrate, rather than an inducer of ATPase activity, was established by chromatography of the

reaction mixture in which 2,5-anhydro-D-mannitol 1,6-P₂ was identified by its R_F compared to an authentic sample. The Lineweaver-Burk plots for both fructose 6-P and 2,5-anhydromannitol 1-P are depicted in Fig. III-6 for comparison. The Michaelis constant for this analogue was found to be 0.41 mM as compared to 0.043 mM for fructose 6-P. The V_{max} for the former was 190 and for the latter was 217 μ moles/min/mg protein, i.e., the maximal velocity for the analogue was 87% of that of the natural substrate. This is the highest V_{max} reported for an alternate substrate of phosphofructokinase. This observation is all the more significant since only four other compounds have been found to be acted upon by this very selective enzyme. These alternate substrates and their V_{max} (relative to fructose 6-P) are: D-tagotose 6-P, 50% (51); D-fructose 1-P, 5% (52); α -D-glucopyranosyl 1-P, 0.67% (53); and D-sedoheptulose 7-P, whose V_{max} was not determined (54).

2,5-Anhydro-D-glucitol 6-P was found to be an effective competitive inhibitor of phosphofructokinase with a K_i of 0.34 mM (Fig. III-7). This inhibition constant is lower than that of the other competitive inhibitors of this enzyme, namely D-fructose 1-P (K_i = 13 mM) (52) and D-arabinose 5-P (K_i = 1.0 mM) (Chapter IV).

Several other analogues failed to show any activity as alternate substrates or inhibitors. These compounds (and the highest levels at which we tested them) are as

Fig. III-6. Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-P and 2,5-anhydro-D-mannitol 1-P as a function of substrate concentration. Assay conditions are as described in "MATERIALS AND METHODS" (Section B), Part 5 of this chapter.

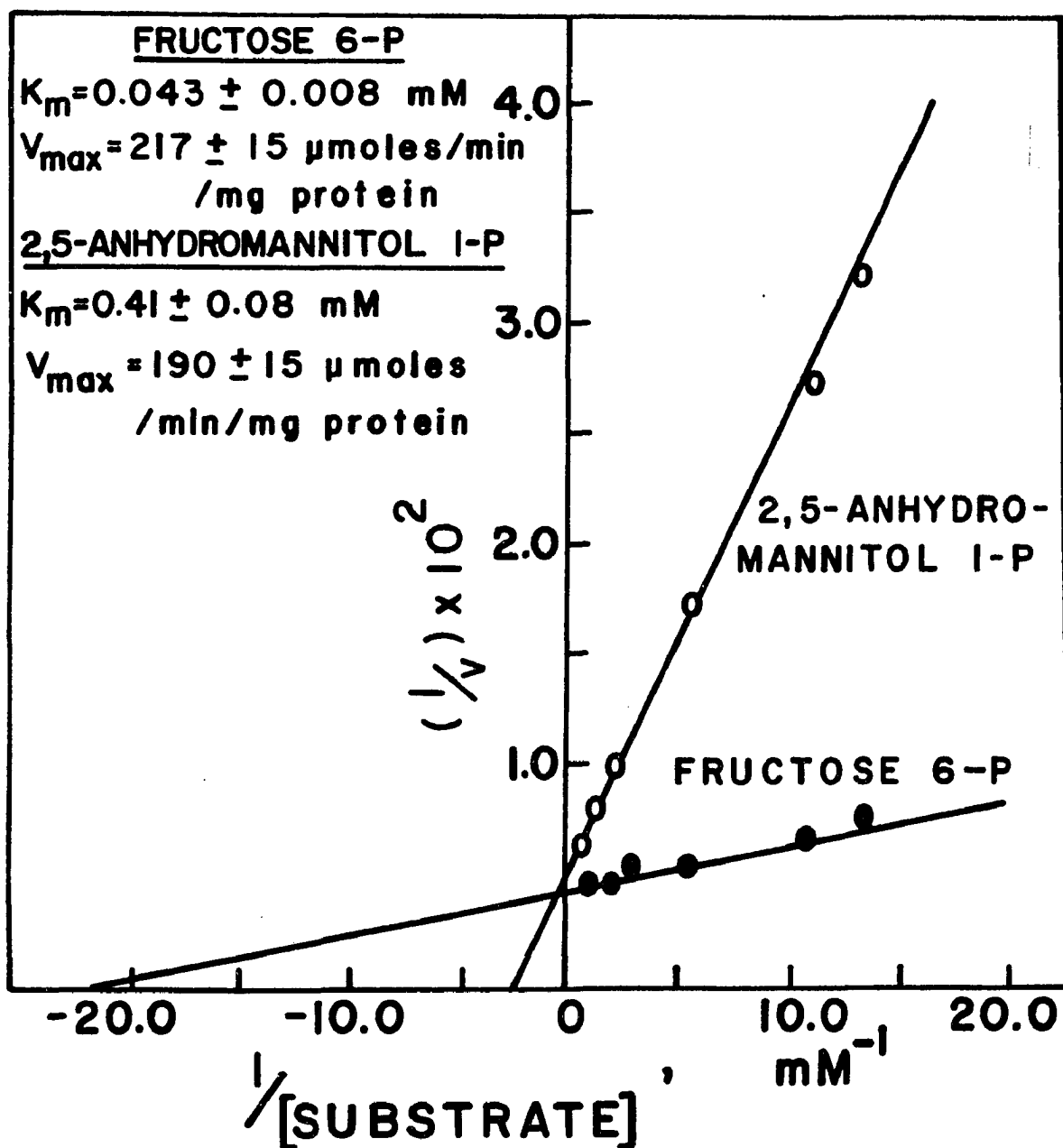
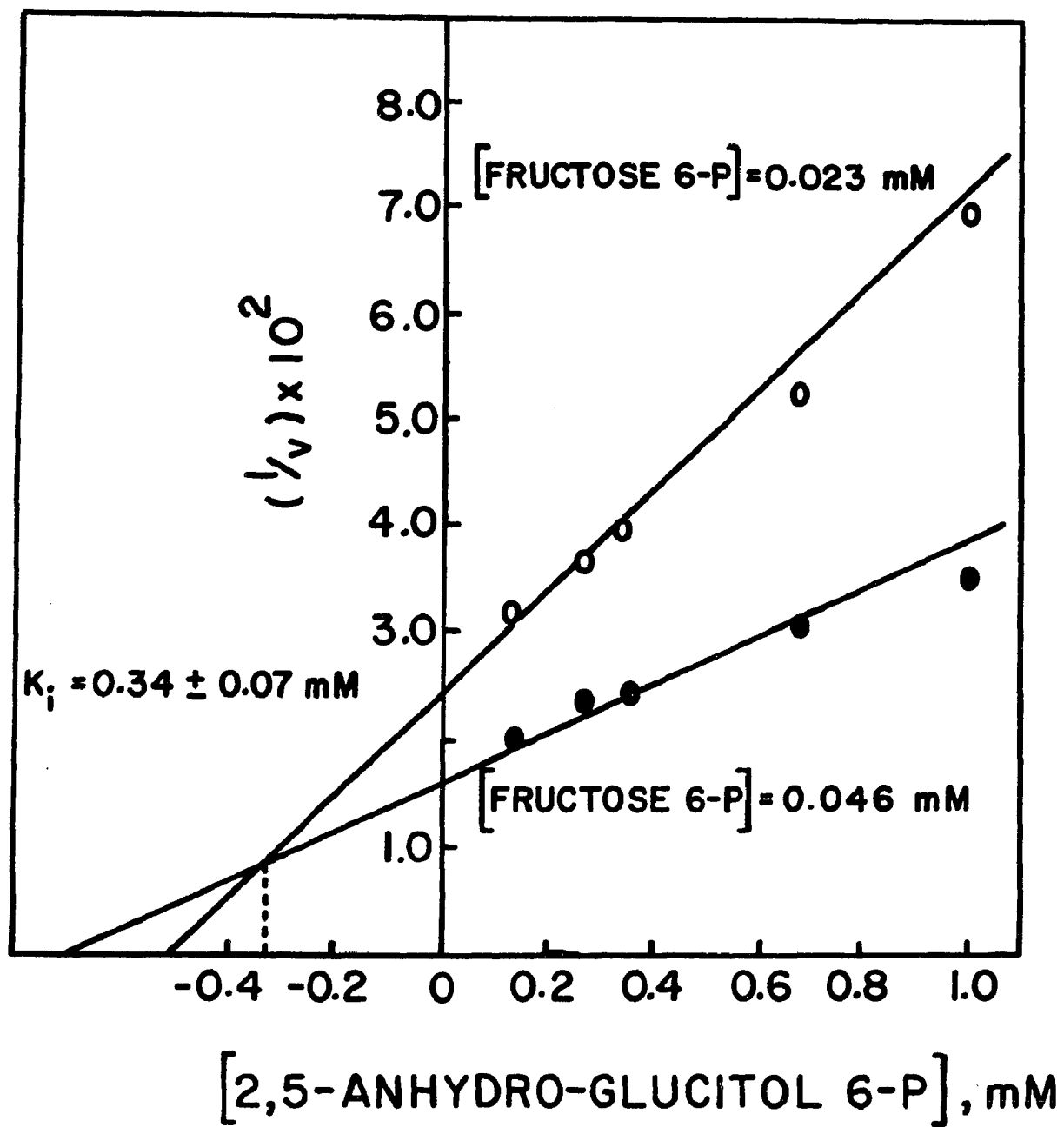


Fig. III-7. Dixon plot of the competitive inhibition of phosphorylation of D-fructose 6-P by 2,5-anhydro-D-glucitol 6-P. Assay conditions are as described under "MATERIALS AND METHODS" (Section B), Part 5 of this chapter.



follows: D-mannitol 1-P, IV (4.0 mM); D-glucitol 6-P, V (2.0 mM); D-xylulose 5-P, VI (4.0 mM); and dihydroxyacetone (200 mM).

D. Discussion

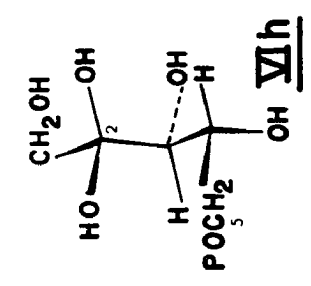
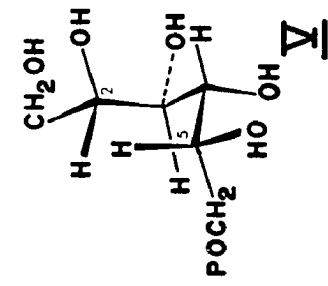
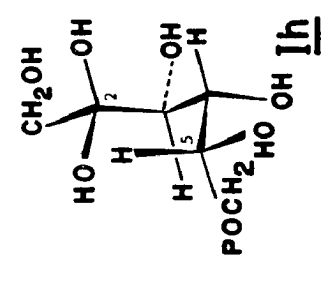
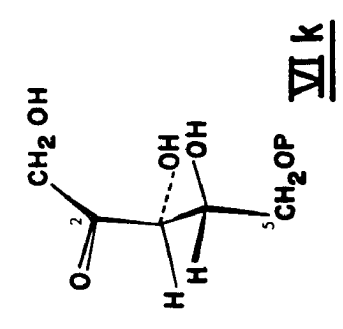
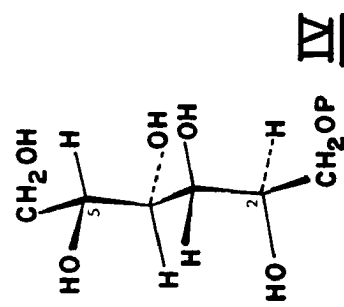
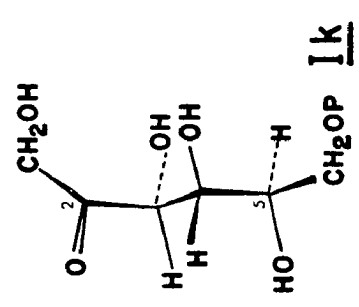
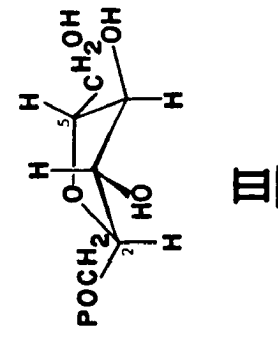
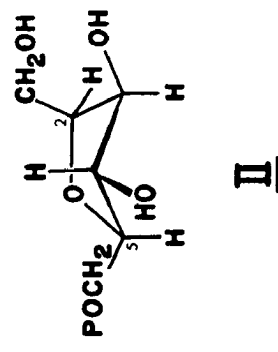
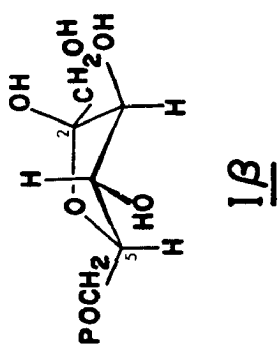
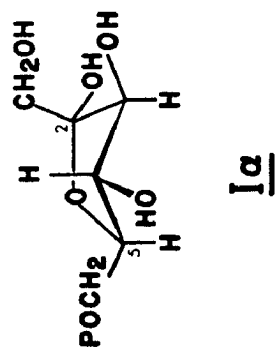
1. Suitability of Analogues

In order to assess effectively the enzymatic importance of each of the four forms of fructose 6-P, the analogues to be used should mimic as closely as possible the total stereochemistry of each form of the substrate. Aspects of stereochemistry³ that should be considered in selecting these analogues include the constitution, configuration and conformation of each form as it exists in solution. Moreover, each analogue must have a fixed structure incapable of interconversion into structures that would resemble the other forms of fructose 6-P in solution. The analogues used in this study were chosen to satisfy these requirements (Fig. III-8).

2,5-Anhydro-D-glucitol 6-P (II) was selected as an analogue of the α -anomer of fructose 6-P (I α). The constitution and configuration at each chiral center of II and I α are identical except at C-2 where II differs from I α in loss of the anomeric oxygen. Though slight, this permutation completely prevents tautomerization or

³ Most stereochemical terms are defined as in reference no. (66).

Fig. III-8. Probable conformations of the tautomeric and anomeric forms of D-fructose 6-phosphate (I α , I β , I k , and I h) and their analogues (II-VI) in solution. Top: α -D-fructofuranose 6-P (I α), β -D-fructofuranose 6-P (I β), keto-D-fructose 6-P (I k) and hydrated keto-D-fructose 6-P (I h). Middle: 2,5-anhydro-D-glucitol 6-P (II), 2,5-anhydro-D-mannitol 1-P (III), D-mannitol 1-P (IV) and D-glucitol 6-P (V). Bottom: keto-D-xylulose 5-P (VI k) and hydrated keto-D-xylulose 5-P (VI h). To be noted is the reversed numbering pattern in III and IV. The conformations in which the above compounds are depicted are: I α , I β , II, and III, 3E conformation; I k , IV, and VI k , planar zig-zag conformation; and I h , V, VI h , sickle conformation. I β and III might also exist in the 3T_2 conformation, a closely related derivative of the 3E conformation. Conformational terms and symbols are in accord with recent usage (66, 67).



anomerization of II. An equally important consideration is that these two sugar phosphates probably exist in the same conformation (Fig. III-8) in solution. Thus II is isosteric in every aspect with I α . A parallel argument leads to the conclusion that 2,5-anhydro-D-mannitol 1-P² (III) is a structurally-locked, isosteric analogue of the β -anomer of fructose 6-P (I β).

D-Mannitol 1-P² (IV), D-glucitol 6-P (V), D-xylulose 5-P (VI) and dihydroxyacetone at high concentration were chosen as analogues of the acyclic keto form of fructose 6-P (I k) and its hydrate (I h). Each of these analogues is structurally fixed in an acyclic structure and is isosteric with the key structural features of the keto form in one of its following four possible modes of binding the enzyme:

(a) Assuming that the C-2 functional group is the dominant binding functionality (e.g. carbonyl of I k forms a Schiff base, or gem-diol of I h forms two key hydrogen-bonds), then dihydroxyacetone in very high concentration is a good analogue of I k and I h . (b) Assuming that the planar zig-zag conformation, hydroxyl group configuration and phosphate charge of I k are dominant structural features in binding, then IV is isosteric with I k . (c) Assuming that the sickle conformation, hydroxyl group configuration and phosphate charge of I h are dominant structural features, then V is isosteric with I h . (d) Assuming that binding is through the structural features assumed in both (a) and (b) or both (a) and (c), then a good compromise is

D-xylulose 5-P since it exists in solution as both a keto form (VIk) in the planar zig-zag conformation and a minor hydrated keto form (VIh) probably in the sickle conformation (Fig. III-8).

2. Interpretation of the Data on Phosphofructokinase Activity

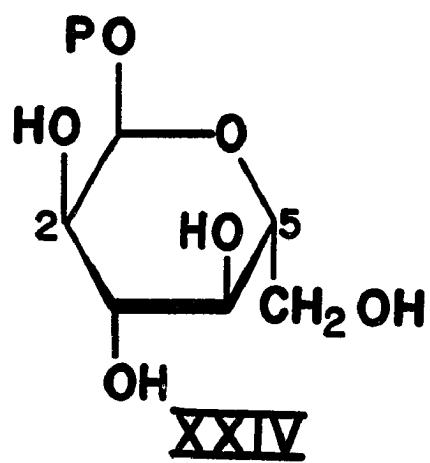
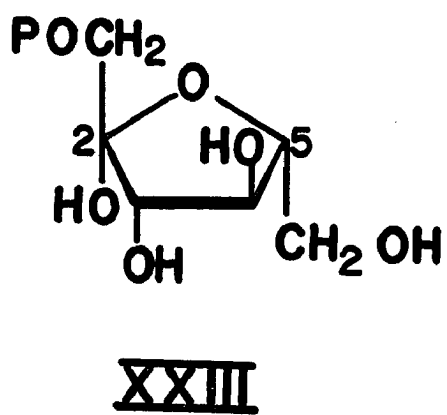
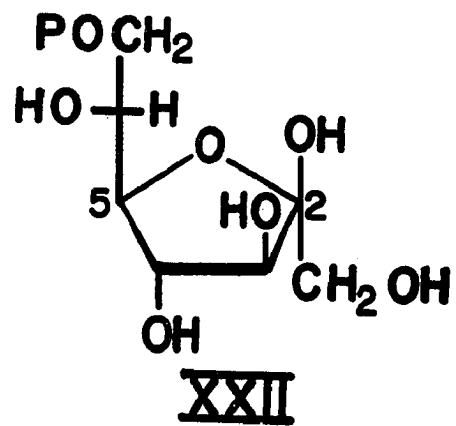
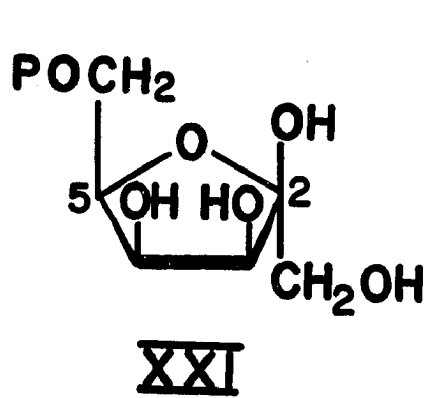
Since the analogues of the cyclic furanose forms of fructose 6-P (II and III) were bound by rabbit muscle phosphofructokinase and the analogues of the acyclic keto form and its hydrate (IV, V and VI) were not, it is concluded that, in binding, the fructose 6-P site has a tautomeric specificity for the cyclic furanose forms of its substrate. The observation that only the analogue of the β -anomer (III) was phosphorylated and displayed kinetic constants similar to those of the natural substrate indicates that, in phosphorylation, the fructose 6-P site has a more restrictive anomeric specificity, acting only on β -D-fructofuranose 6-P.

The conclusion that phosphofructokinase phosphorylates only the β -anomer of fructose 6-P is further strengthened by a comparison of the binding constants of 2,5-anhydromannitol 1-P and 2,5-anhydroglucitol 6-P. The former, the β -anomer analogue, has a K_m of 0.41 mM, whereas the latter, the α -anomer analogue, has a K_i of 0.34 mM. The near equality of K_m and K_i of these two synthetic analogues indicates that the enzyme has almost equal affinity for

them. It should be noted that the only structural difference between them is the configuration around one carbon, namely C-5 of the anhydromannitol analogue (III) and C-2 of the anhydroglucitol analogue (II). These are the carbons that correspond to the anomeric C-2 carbon of the fructofuranose ring ($I\alpha$ and $I\beta$). Apparently, the mere shift from the β -form to the α -form puts the C-1 hydroxymethyl group in a position unfavorable for its phosphorylation and transforms the compound from a substrate to a competitive inhibitor.

Moreover, our proposal that the β -furanose form is the only active substrate form explains the enzymatic activities of all other known alternate substrates of phosphofructokinase (Fig. III-9). D-Tagatose 6-P, a good alternate substrate of the enzyme with a V_{\max} half that of the natural substrate (51), exists in solution predominantly in its furanose tautomer, of which at least 60% is probably β -anomer. D-Sedoheptulose 7-P, another known alternate substrate (54) exists significantly in its furanose tautomer, of which approximately 25% is probably β -anomer. D-Fructose 1-P, whose K_m is 3.8 mM and relative V_{\max} is 5% (52), has been shown through ^{13}C -NMR spectroscopy (55) to be composed of approximately 15% β -fructofuranose 1-P. This β -furanose form has a structure that is almost superimposable on β -fructofuranose 6-P when rotated 180° . The only difference being the C-2 hydroxyl of one structure is positioned at the C-5 of the other structure. When

Fig. III-9. Other known alternate substrates of phospho-fructokinase: D-tagatose 6-phosphate (XXI), D-sedoheptulose 7-phosphate (XXII), D-fructose 1-phosphate (XXIII), and α -D-glucopyranosyl 1-phosphate (XXIV). Sugars capable of tautomerization and anomerization have been drawn in their β -furanose form.



corrections are made for its low concentration and improperly positioned C-2 hydroxyl, the β -furanose form accounts well for the observed kinetic constants of fructose 1-P. Finally, α -D-glucopyranosyl 1-P, a very poor alternate substrate of phosphofructokinase with a relative V_{\max} of 0.67% (53), does not exist as its β -furanose form in solution since it is covalently locked in its α -pyranose form. The weak activity observed with this sugar phosphate can be explained by the fact that when this molecule is rotated 180°, it provides some semblance of β -fructofuranose 6-P.

Recently, Wurster and Hess (56) arrived at a conclusion identical to ours, using a completely different approach, namely stopped flow kinetic technique, to study the anomeric specificity of phosphofructokinase. They observed that approximately 76% of the equilibrium mixture of fructose 6-P reacted in a fast initial phase which was followed by a slower one. Since it had been shown by ^{13}C NMR spectroscopy that fructose 6-P exists as 80% β -anomer and 20% as α -anomer in solution (7, 57), Wurster and Hess concluded that phosphofructokinase acts on the β -form of its substrate. They explained the slower phase of the reaction as representing the spontaneous anomerization of the α - to the β -form of fructose 6-P.

The evidence presented in this communication substantiates our earlier proposal (7, 8) that the β -anomer is the true substrate of phosphofructokinase, but does not

support the suggestion of Schray et al. (10) and Benkovic et al. (11) that the α -anomer of fructose 6-P can be phosphorylated by this enzyme. Moreover, evidence in the literature tends to indicate that phosphofructokinase from other sources might also be specific for the β -anomer of fructose 6-P. Thus, Sols and Salas (58) have noticed that 2,5-anhydro-D-glucitol 6-P, the α -anomer analogue, was an inhibitor but not a substrate of yeast phosphofructokinase. In view of these findings, we propose that phosphofructokinase be more accurately renamed ATP: β -D-fructofuranose 6-phosphate 1-phosphotransferase.

As mentioned above, we reported (7) that the β -anomer is the predominant form of fructose 6-P in solution. The specificity of phosphofructokinase for this form of the substrate could have resulted from a natural selection of an acceptor site capable of binding and phosphorylating the anomer most abundant in solution. In this regard, the evolutionary path of phosphofructokinase seems to be similar to that of aldolase which acts specifically on β -D-fructofuranose 1,6-P₂ (59). However, it seems that aldolase, like phosphofructokinase, can bind forms other than the one it acts on. Thus, Hartman and Barker (60) have shown that structural analogues of all forms of fructose 1,6-P₂ bind aldolase with similar affinities.

Finally, we would like to note that tautomeric and anomeric specificity might play a regulatory role and that several enzymes of carbohydrate metabolism manifest such

specificity. Among these are microbial glucose 6-phosphate dehydrogenase (61), glucose 6-phosphate isomerase (61, 62), mannose 6-phosphate isomerase (63), pentose isomerases (64) and bovine liver fructokinase (65).

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Koerner, T. A. W., Jr., Younathan, E. S., Ashour, A. E., and Voll, R. J. (1974) J. Biol. Chem. 249, 5749.

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CHAPTER IV
AN EXPLORATION OF THE FRUCTOSE 6-PHOSPHATE SITE
OF PHOSPHOFRUCTOKINASE.
EPIMERIC SPECIFICITY

A. Introduction

In the previous chapter and communications (1-5), we have proposed that rabbit muscle phosphofructokinase (EC 2.7.1.11) phosphorylates only the β -anomer of its substrate D-fructose 6-P. We provided experimental evidence for our proposal through probing the fructose 6-P site of this enzyme with isosteric and structurally-locked analogues of all four isomeric forms of fructose 6-P (2, 3). Moreover, we have shown that the fructose 6-P site of phosphofructokinase has two specificities, namely, a tautomeric specificity for the cyclic furanose form (to bind the ligand as substrate or inhibitor) and a more restrictive anomeric specificity for the β -D-furanose form (to catalyze the transphosphorylation reaction).

Using approaches similar to ours, Bar-Tana and Cleland (6), and Fishbein et al. (7) arrived at similar conclusions. Further support for this contention came from the findings of Wurster and Hess (8) using a fast flow kinetic technique.

After establishing the tautomeric and anomeric (C-2 configurational) specificities of phosphofructokinase, we investigated the configurational specificity of this

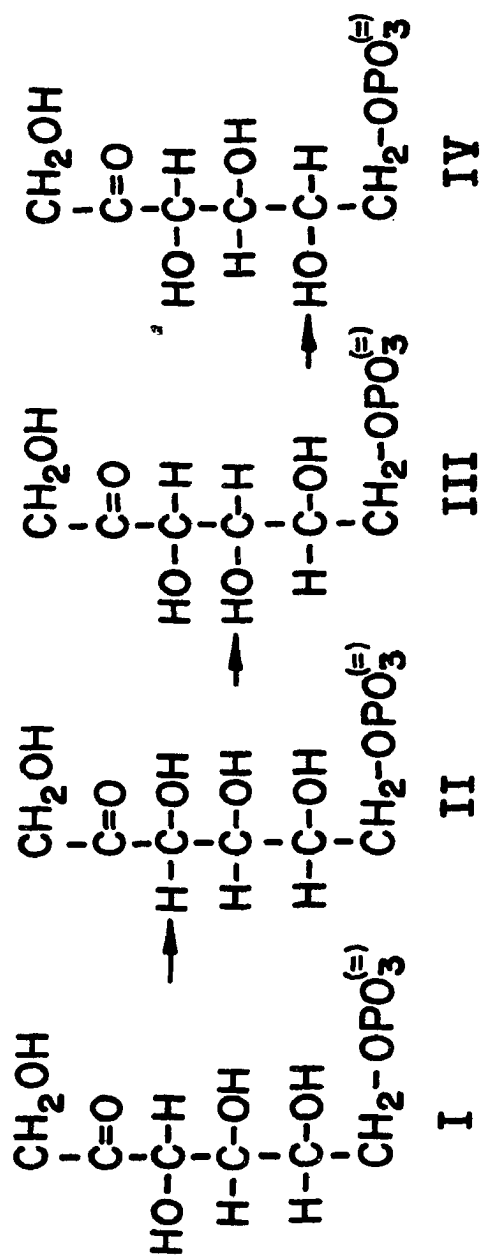
enzyme at the remaining three asymmetric carbons of fructose 6-P, namely C-3, C-4, and C-5. In this study we tested as alternate substrates sugar phosphates which are epimeric to D-fructose 6-P, i.e., stereochemically identical to the physiological substrate except in the configuration around one of these three asymmetric carbons (9). These epimers are (Fig. IV-1): D-psicose 6-P (C-3 epimer), D-tagatose 6-P (C-4 epimer), and L-sorbose 6-P (C-5 epimer). We have synthesized pure samples of each of these three sugar phosphates and established their structure by physical methods. Early studies on the epimeric specificity of phosphofructokinase were carried out by Lardy and his co-workers (10-13).

B. Materials and Methods

1. Commercial Materials

D-Fructose, D-tagatose, ATP, NADH, phosphoenol pyruvate, dithiothreitol, pyruvate kinase, and lactate dehydrogenase were purchased from Sigma Chemical Co. Ruthenium dioxide, sodium borohydride, and platinum oxide were products of Ventron-Alfa Inorganics, Inc. Diphenyl phosphochloridate was obtained from Aldrich Chemical Co. 2,3:4,6-Di-O-isopropylidene- α -L-sorbofuranose was generously provided by Dr. W. E. Scott of Hoffmann-La Roche, Inc., and Dr. Norman G. Brink of Merck Sharp and Dohme, Inc.

Fig. IV-1. The Fischer projection formulae of D-fructose 6-P (I) and its epimers: D-psicose 6-P (II), D-tagatose 6-P (III) and L-sorbose 6-P (IV). The systematic names of these ketose phosphates are: D-arabino-hexulose 6-P (I), D-ribo-hexulose 6-P (II), D-lyxo-hexulose 6-P (III) and L-xylo-hexulose 6-P (IV), respectively.



D-FRUCTOSE 6-P D-PSICOSE 6-P D-TAGATOSE 6-P L-SORBOSE 6-P

ARROW INDICATES EPIMERIC CARBON

2. General Procedures

L-Sorbitose 6-P was prepared by the method of Mann and Lardy (11). D-Tagatose 6-P was synthesized by the method of Totten and Lardy (10) with some modifications (vide infra) that led to higher intermediate and overall yields. The novel D-psicose 6-P was prepared by a chemical method which is described under RESULTS. All three ketose phosphates and their synthetic precursors were thoroughly characterized by several physical and chemical methods. Sugar phosphates, synthesized as barium salts, were converted to the corresponding disodium salts for enzymatic studies and to diammonium salts for paper chromatography by treatment with an equivalent amount of the appropriate sulfate and removal of the barium sulfate by centrifugation.

Solvent 3 was composed of benzene-absolute methanol, 24:1 by volume. Acetone and pyridine were dried by storage over Linde 4A molecular sieve. Other materials, solvent mixtures, and details of analytical and synthetic procedures were as we reported in a previous communication (3) and Chapter III.

Catalytic hydrogenations were conducted in a Parr series 3910 hydrogenation apparatus (Fig. IV-2). Runs were routinely carried out at 2-3 atm. of pressure. Hydrogenolysis of phenyl blocking groups from diphenyl phosphate triesters was effected through platinum-catalysis according

Fig. IV-2. Drawing of the Parr series 3910 hydrogenation apparatus, 500 ml, low pressure, shaker-type with motor and switch on steel base plate.
(Drawing courtesy Parr Instrument Co., Moline, Illinois)

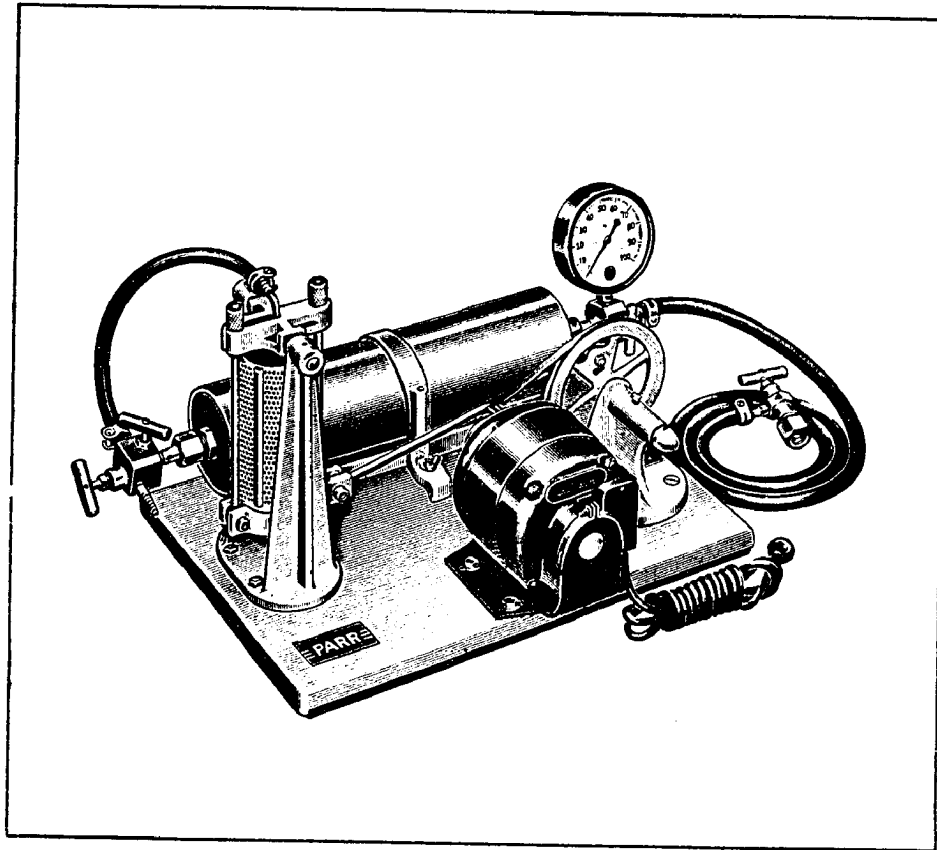
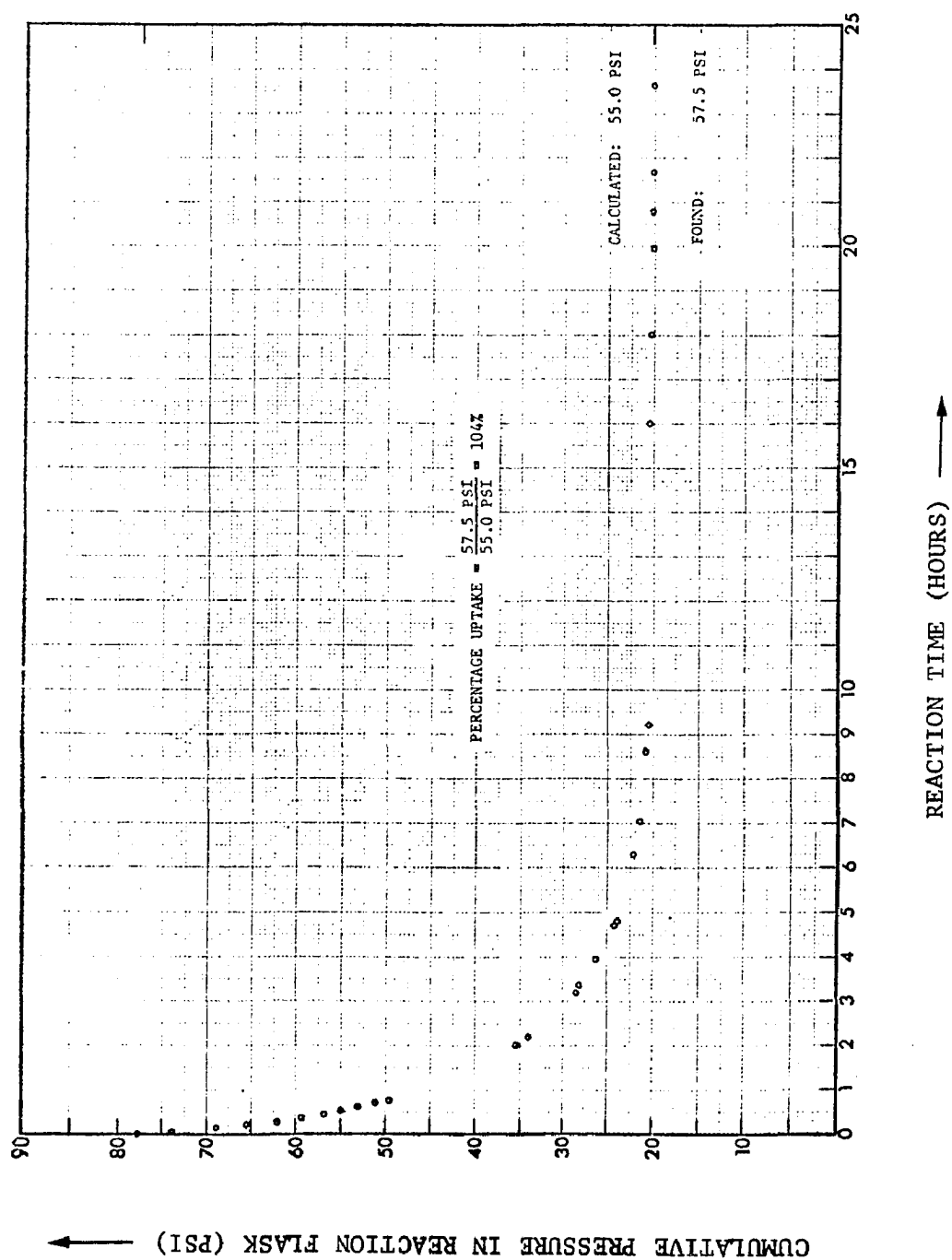


Fig. IV-3. Kinetics of an exemplary catalytic hydrogenation and computation of the percentage uptake of hydrogen gas.



to the general procedure of Brigl and Muller (14). The progress of hydrogenation reactions was monitored by plotting the decrease in the reaction vessel pressure as a function of time (Fig. IV-3).

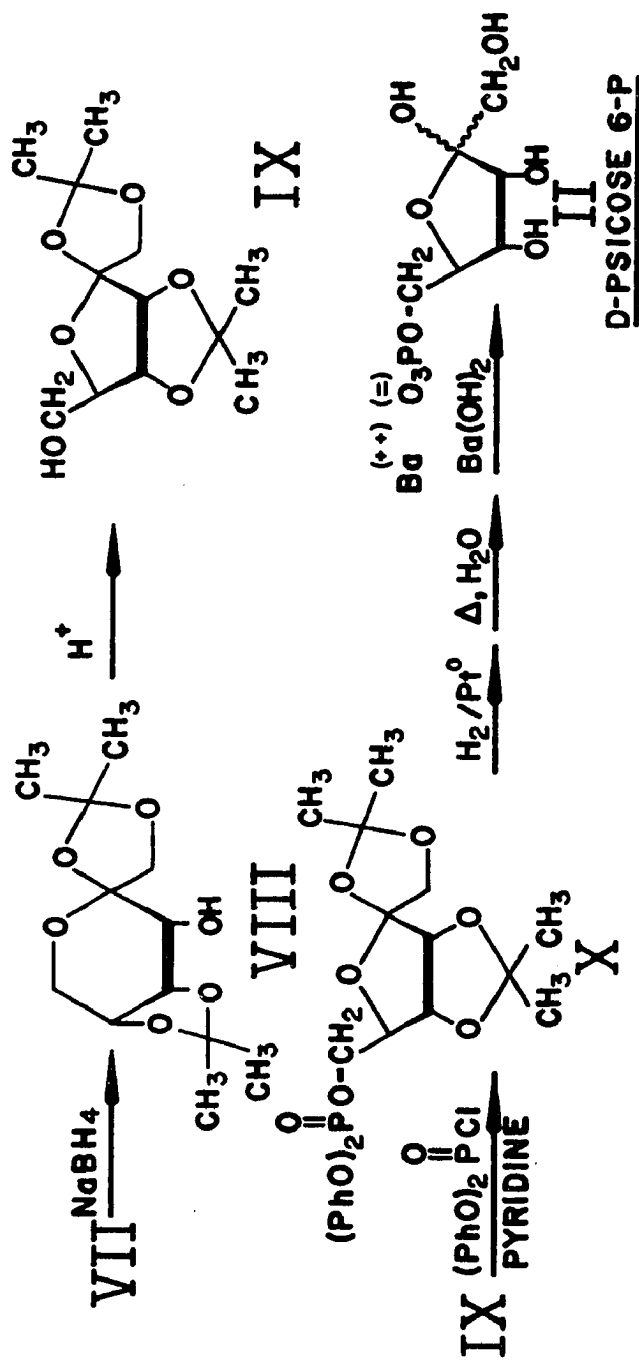
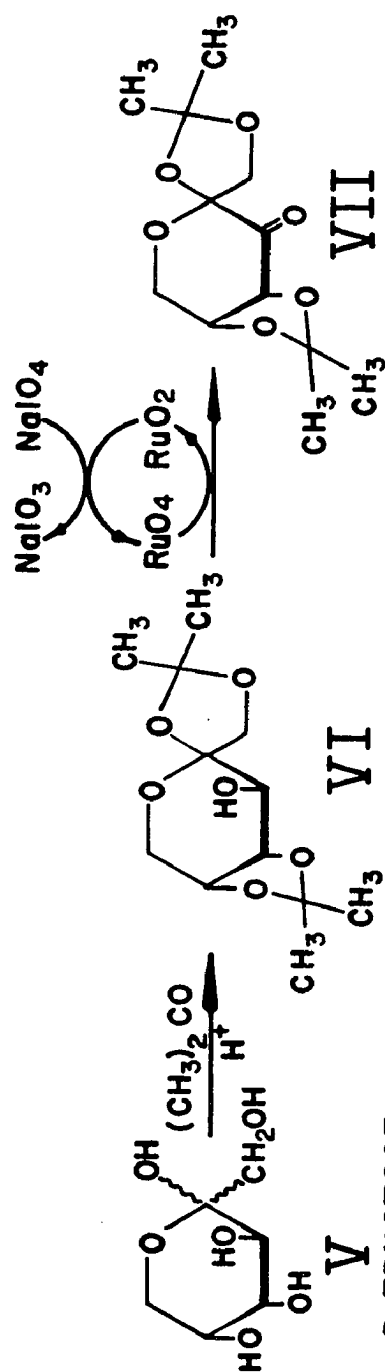
Infrared spectra were obtained with a Perkin-Elmer Model 137 spectrophotometer using potassium bromide pellets of the samples. Optical rotations were determined with a JASCO J-20 recording spectropolarimeter. Nuclear magnetic resonance spectra were recorded on Varian A-60 and HA-100 spectrometers. UV spectra generated in the enzymatic assay (infra vide) were recorded on a Gilford recording spectrophotometer.

3. Synthesis of 1,2:3,4-Di-O-isopropylidene- β -D-psicofuranose (IX)

This diacetal was obtained through a four-step synthesis (V→IX, Fig. IV-4) from D-fructose (V) according to literature procedures (15, 16).

1,2:4,5-Di-O-isopropylidene- β -D-fructopyranose (VI)—was prepared from D-fructose (V) by the improved procedure of Brady (15). Recrystallization from ether-hexane afforded long, rectangular plates, m.p. 118-119°C; literature (15) m.p. 119°C. TLC analysis in solvent 3 showed a single spot, R_F 0.21, identical to that in the literature (15). The proton NMR spectrum of VI (DCCl_3 ; TMS, 100 MHz) was identical to that reported in the literature (17) (Tables VI-1 and VI-2).

Fig. IV-4. Synthesis of D-psicose 6-P (II) from D-fructose
(V) .



1,2:4,5-Di-O-isopropylidene- β -D-erythro-2,3-hexodiulo-2,6-pyranose (VII) — was obtained from VI by ruthenium tetroxide-potassium periodate oxidation, according to the method of Tipson et al. (16). Recrystallization from ether-petroleum ether gave rosettes of long thin platelets, m.p. 100-103°C; literature, m.p. 102-103°C. TLC analysis in Solvent 3 showed the product to be a single component, R_F 0.63: literature (16), R_F 0.65 in Solvent 3. IR data (λ_{max}): 5.75 (C=O), 7.24 and 7.26 [doublet, C(CH₃)₂], and all other absorptions were identical with those as reported in the literature (16). The proton NMR spectrum was consistent with the assigned structure (Tables IV-1 and IV-2 and Fig. IV-5).

1,2:4,5-Di-O-isopropylidene- β -D-psicopyranose (VIII) — was prepared from the ketone VII by the sodium borohydride reduction according to the procedure of Tipson et al. (16). Recrystallization from hexane yielded clear, rectangular plates, m.p. 67-68°C; literature (16), m.p. 68-69°C. TLC analysis in Solvent 3 indicated the product was pure, R_F 0.20; literature (16), R_F 0.21 in Solvent 3. The proton NMR spectrum was consistent with the assigned structure (Tables IV-1 and IV-2 and Fig. IV-6).

1,2:3,4-Di-O-isopropylidene- β -D-psicofuranose (IX) — was obtained through the acid-catalyzed isomerization of VIII, according to the procedure of Brady (15). Crystallization from hexane and slow recrystallization from slightly

TABLE IV-I

PROTON CHEMICAL SHIFT DATA FOR DI-O-ISOPROPYLIDENE HEXULOSE DIPHENYL-PHOSPHATES AND THEIR PRECURSORS, MEASURED AT 100 MHz in DEUTERIO-CHLOROFORM

| Compound | Structure | Chemical shifts (δ) in deuterio-chloroform | | | | | | | | | | | |
|--|-----------|---|------|------|------|---------------|------|------|------------------------|------------|--------|------|--|
| | | H-1 | H-1' | H-3 | H-4 | H-5 | H-6 | H-6' | Isopropylidene | Methyls*** | Phenyl | OH | |
| 1,2:3,4-di-O-isopropylidene α -D-tagatofuranose | XI | 4.30 | 4.08 | 4.64 | 4.86 | 4.10 | 3.90 | 3.89 | 1.49, 1.45, 1.42, 1.33 | --- | --- | 2.42 | |
| 1,2:3,4-di-O-isopropylidene α -D-tagatofuranose 6-diphenylphosphate | XIII | 4.27 | 4.03 | 4.58 | 4.76 | 4.28 | 4.51 | 4.45 | 1.42, 1.38(6), 1.26 | 7.02-7.41 | --- | --- | |
| 1,2:4,5-di-O-isopropylidene β -D-fructopyranose* | VI | 4.18 | 3.98 | 3.67 | 4.2 | ** | ** | 4.0 | 1.55, 1.54, 1.46, 1.39 | --- | --- | 2.09 | |
| 1,2:4,5-di-O-isopropylidene β -D-erythro-2,3- hexodiulo-2,6-pyranose | VII | 4.60 | 3.98 | -- | 4.72 | 4.54 | 4.40 | 4.10 | 1.55, 1.46, 1.40(6) | --- | --- | --- | |
| 1,2:4,5-di-O-isopropylidene β -D-psicopyranose | VIII | 4.26 | 4.03 | 3.76 | 4.44 | 4.22 | 4.00 | 3.99 | 1.55, 1.50, 1.40, 1.37 | --- | --- | 2.46 | |
| 1,2:3,4-di-O-isopropylidene β -D-psicofuranose | IX | 4.34 | 4.05 | 4.63 | 4.88 | 4.27 | 3.76 | 3.60 | 1.51, 1.45, 1.41, 1.33 | --- | --- | 3.03 | |
| 1,2:3,4-di-O-isopropylidene β -D-psicofuranose 6-diphenylphosphate | X | 4.27 | 4.02 | 4.54 | 4.68 | 4.19- 4.27 | 4.24 | 4.24 | 1.42, 1.39, 1.35, 1.28 | 7.02-7.40 | --- | --- | |
| 2,3:4,5-di-O-isopropylidene β -D-fructopyranose* | | 3.69 | 3.65 | 4.34 | 4.63 | 4.23 | 3.91 | 3.76 | 1.55, 1.47, 1.40, 1.35 | --- | --- | 2.41 | |
| 2,3:4,5-di-O-isopropylidene β -D-fructopyranose 1-diphenylphosphate | | 4.37 | 4.23 | 4.37 | 4.60 | 4.21 | 3.93 | 3.73 | 1.50, 1.44, 1.32, 1.28 | 7.02-7.40 | --- | --- | |

* values reported in Ref. 17

** not reported because obscured

***all methyl peaks were 3-proton singlets except where noted in parenthesis

TABLE IV-2

FIRST-ORDER COUPLING-CONSTANTS FOR DI-O-ISOPROPYLIDENE HEXULOSE DIPHENYL-PHOSPHATES AND THEIR PRECURSORS, MEASURED AT 100 MHZ IN DEUTERIO-CHLOROFORM

| Compound | Structure | Coupling constants (absolute values in Hz, estimated error ± 0.2 Hz) | | | | | | | | | | |
|--|-----------|--|-----------|-----------|-----------|------------|------------|-----------|------------|-----------|------------|------------------------------------|
| | | $J_{1,1'}$ | $J_{3,4}$ | $J_{4,5}$ | $J_{5,6}$ | $J_{5,6'}$ | $J_{6,6'}$ | $J_{1,P}$ | $J_{1',P}$ | $J_{6,P}$ | $J_{6',P}$ | Other J |
| 1,2:3,4-di-O-isopropylidene α -D-tagatofuranose | XI | 9.5 | 6.0 | 3.5 | 5.0 | 6.0 | <0.1 | -- | -- | -- | -- | $J_{6,OH} = J_{6',OH} = 6.0$ |
| 1,2:3,4-di-O-isopropylidene α -D-tagatofuranose 6-diphenylphosphate | XIII | 9.5 | 5.8 | 3.5 | 5.0 | 6.0 | <0.1 | -- | -- | 7.5 | 8.0 | $^4J_{5,P} = 9.5$ |
| 1,2:4,5-di-O-isopropylidene β -D-fructopyranose* | VI | 8.2 | 7.8 | 5.0 | ** | ** | ** | -- | -- | -- | -- | $J_{3,OH}$ obscured |
| 1,2:4,5-di-O-isopropylidene β -D-erythro-2,3-hexodiulo-2,6-pyranose | VII | 9.5 | -- | 5.2 | 2.1 | 0.9 | 13.0 | -- | -- | -- | -- | $^4J_{4,6'} = 0.4$ |
| 1,2:4,5-di-O-isopropylidene β -D-psicopyranose | VIII | 9.0 | 3.8 | 6.4 | 2.0 | 2.0 | <0.1 | -- | -- | -- | -- | $J_{3,OH} = 6.6$ |
| 1,2:3,4-di-O-isopropylidene β -D-psicofuranose | IX | 9.7 | 5.9 | 1.0 | 3.0 | 3.8 | 12.5 | -- | -- | -- | -- | $J_{6,OH} = J_{6',OH} = 7.0$ |
| 1,2:3,4-di-O-isopropylidene β -D-psicofuranose 6-diphenylphosphate | X | 10.0 | 5.7 | 0.7 | 3.8 | 3.8 | <0.1 | -- | -- | 5.2 | 5.2 | -- |
| 2,3:4,5-di-O-isopropylidene β -D-fructopyranose* | | 11.6 | 2.5 | 8.0 | 1.5 | 1.0 | 13.0 | -- | -- | -- | -- | $J_{1,OH}, J_{1',OH}$ not reported |
| 2,3:4,5-di-O-isopropylidene β -D-fructopyranose 1-diphenylphosphate | | 10.1 | 2.5 | 7.5 | 2.0 | 1.0 | 12.6 | 5.5 | 5.3 | -- | -- | -- |

* values reported in Ref. 17

** not reported because obscured

Fig. IV-5. Proton NMR spectrum of 1,2:4,5-di-O-isopropylidene β -D-erythro-2,3-hexodiulo-2,6-pyranose (VII) recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton.

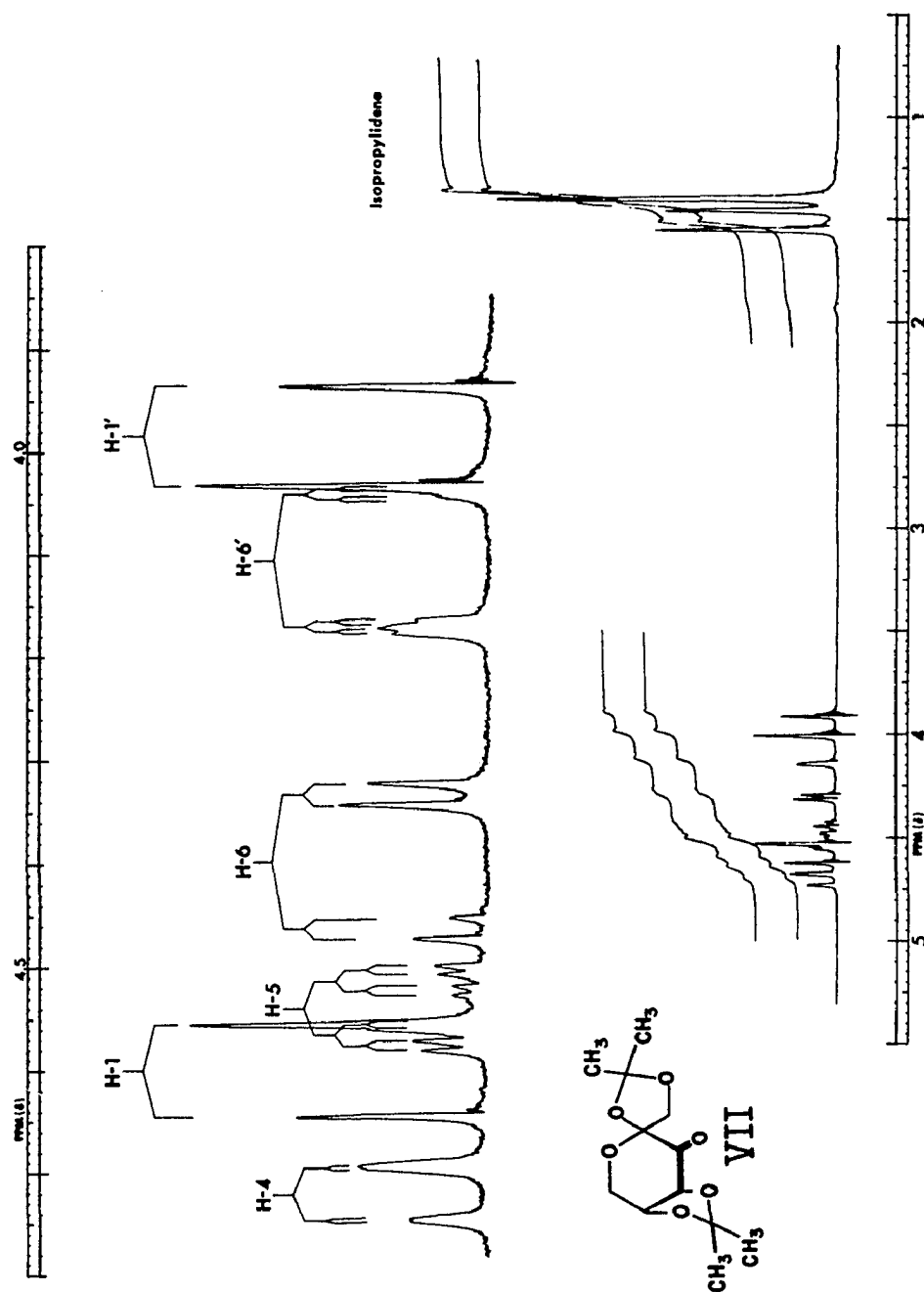
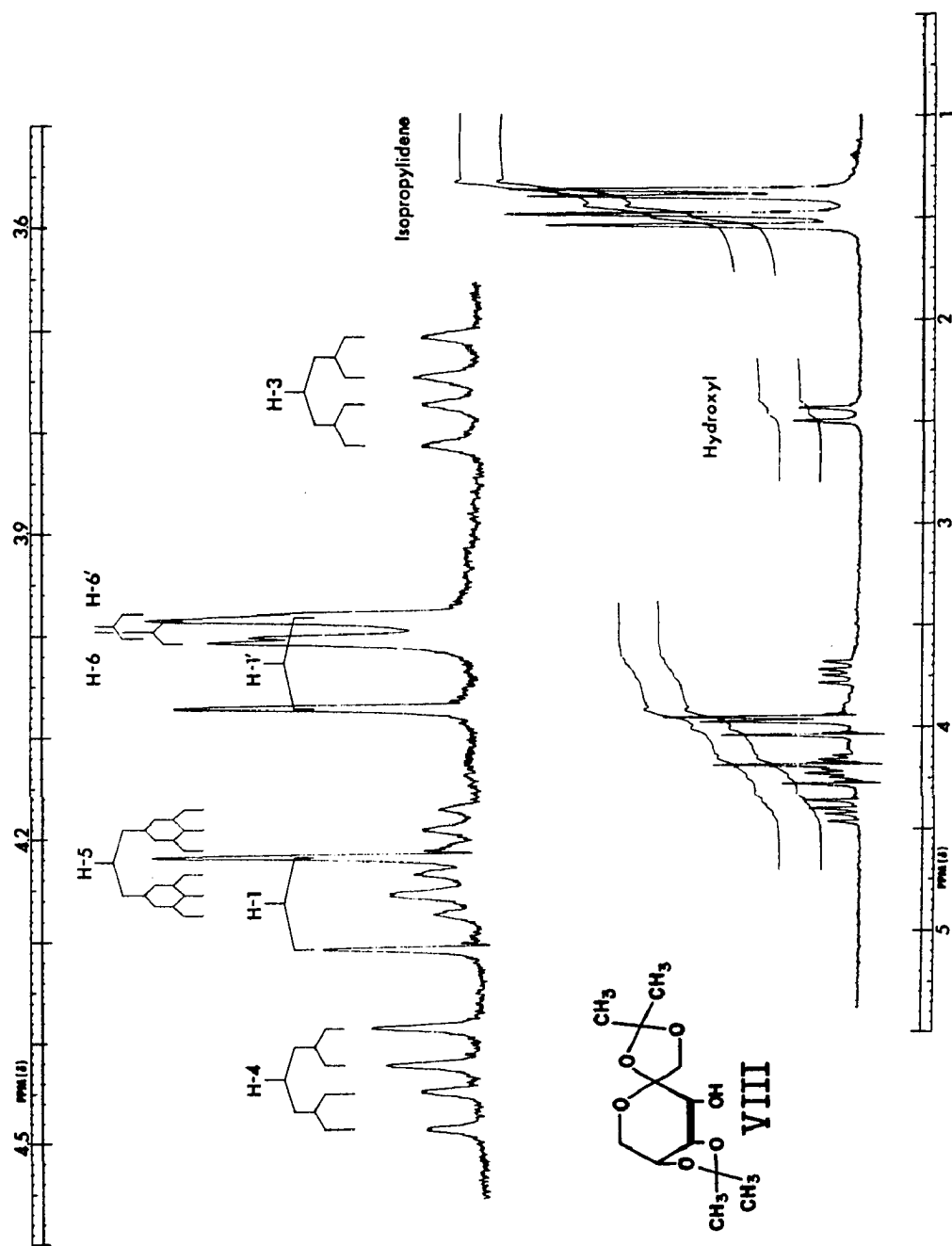


Fig. IV-6. Proton NMR spectrum of 1,2:4,5-di-O-isopropylidene- β -D-psicopyranose (VIII) recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton. The chemical shifts are reported as δ (ppm downfield from tetramethylsilane). Complete analysis of all 20 protons of the molecule resulted in the following data: 1.37, 1.40, 1.50 and 1.55 δ (all four, each a 3-proton singlet, 4 isopropylidene methyls), 2.46 (D_2O -exchangeable 1-proton doublet, hydroxyl, $J_{3,OH}$ 6.6 Hz), 3.76 (1-proton doublet of doublets, H-3, $J_{3,4}$ 3.8 Hz, $J_{3,OH}$ 6.6 Hz), 3.99 (1-proton, A portion of ABX, H-6', $J_{5,6'}$ 2.0 Hz, $J_{6,6'}$ < 0.1 Hz), 4.00 (1-proton, B portion of ABX, H-6, $J_{5,6}$ 2.0 Hz, $J_{6,6'}$ < 0.1 Hz), 4.03 (1-proton, A of AB, H-1', $J_{1,1'}$ 9.0 Hz), 4.22 (1-proton, X of ABX additionally coupled to H-4, H-5, $J_{4,5}$ 6.4 Hz, $J_{5,6}$ and $J_{5,6'}$ 2.0 Hz), 4.26 (1-proton, B of AB, H-1, $J_{1,1'}$ 9.0 Hz), and 4.44 (1-proton doublet of doublets, H-4, $J_{3,4}$ 3.8 Hz, $J_{4,5}$ 6.4 Hz).



alkaline, cold water gave long, rectangular prisms (Fig. IV-7), m.p. 56-57°C, $[\alpha]_D^{25} = 104.9 \pm 0.3^\circ$ (1.00 g per 100 ml acetone); literature values (15) are: m.p. 56-57°C, $[\alpha]_D^{25} = 97.8^\circ$ (0.10 g per 100 ml acetone). TLC analysis in Solvent 3 showed IX to be a single component, R_F 0.20. The proton NMR spectrum was consistent with the assigned structure (Tables IV-1 and IV-2 and Fig. IV-8).

Elemental analysis — for formula $C_{12}H_{20}O_6$ (260.29),

Calculated: C 55.37, H 7.75

Found: C 55.26, H 7.52

4. Synthesis of 1,2:3,4-Di-O-isopropylidene- α -D-tagatofuranose (XI)

This diacetal was obtained from D-tagatose through the isopropylidenization procedure of Reichstein and Bosshard (18). High-vacuum distillation and crystallization from cold hexane and drying under high vacuum gave white needles with physical constants identical to those in the literature (18); m.p. 65-66°C, $[\alpha]_D^{20} = 81.5 \pm 0.2^\circ$ (2.16 g per 100 ml acetone). TLC analysis in Solvent 3 showed the product to be homogeneous (R_F 0.20). The proton NMR spectrum was consistent with the assigned structure (Tables IV-1 and Table IV-2 and Fig. IV-9).

Fig. IV-7. Photograph of crystals of 1,2:3,4-di-O-isopropylidene- β -D-psicofuranose (IX) slowly grown from slightly alkaline water at 4° (magnification 3X). Photography courtesy Mr. R. J. Voll and Mr. R. E. Voll.

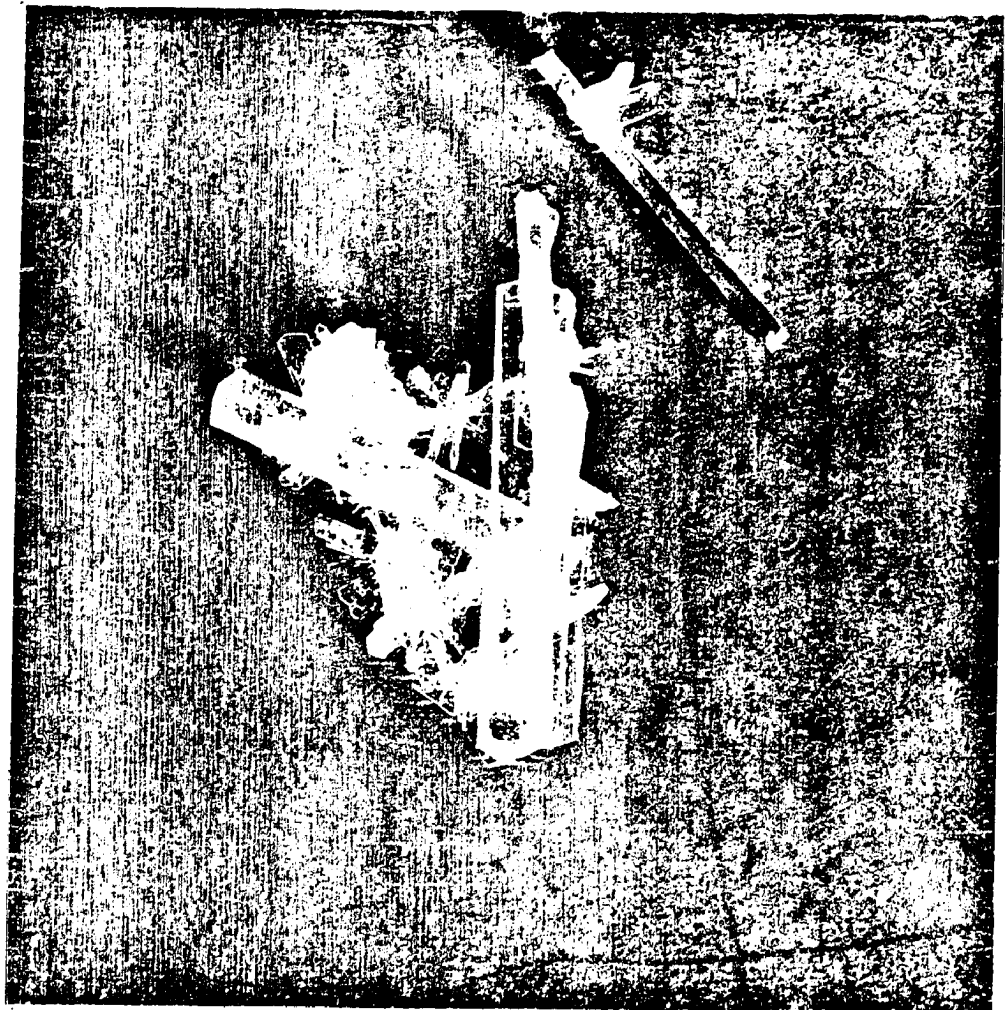


Fig. IV-8. Proton NMR spectrum of 1,2:3,4-di-O-isopropylidene- β -D-psicofuranose (IX) recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton.

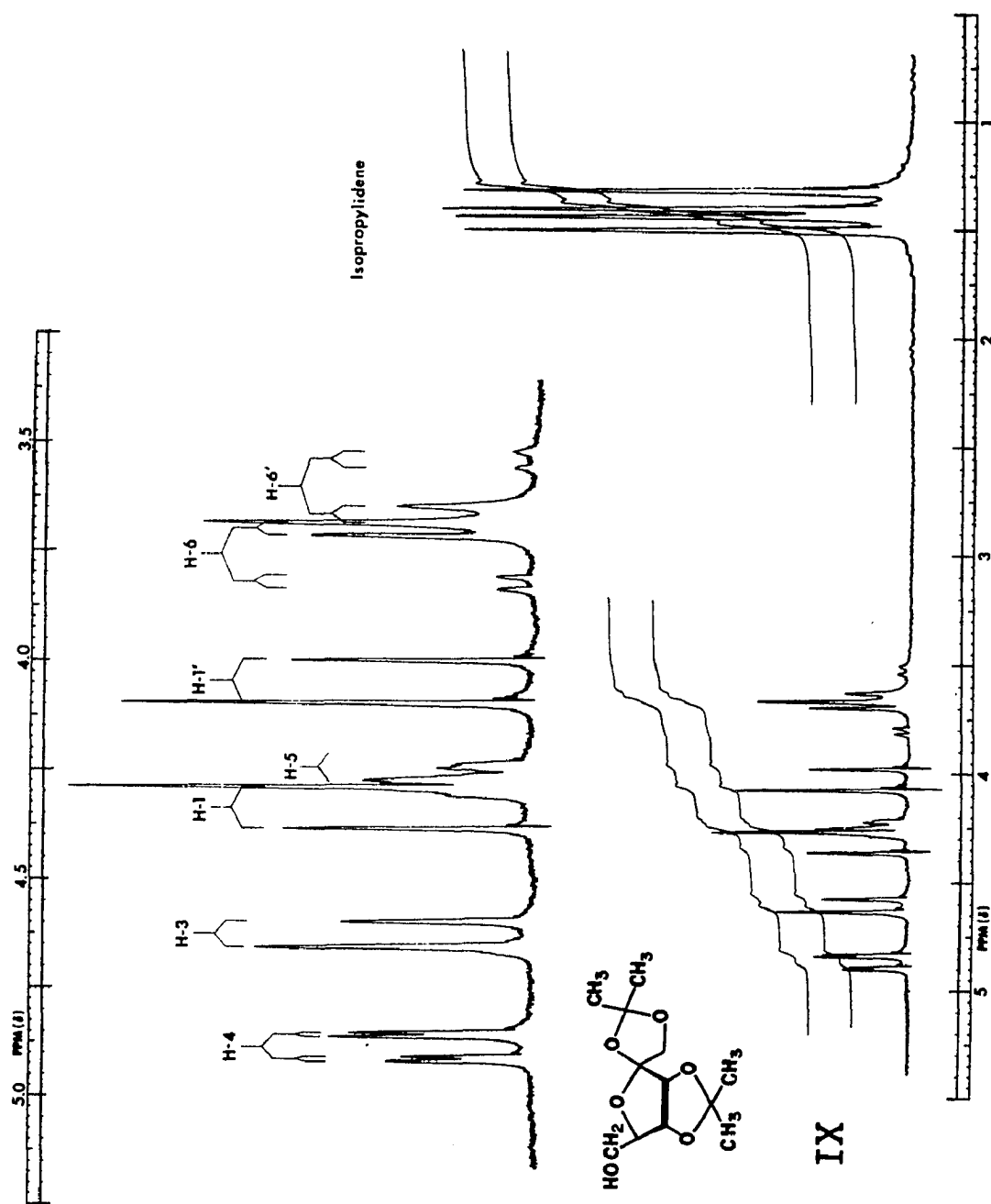
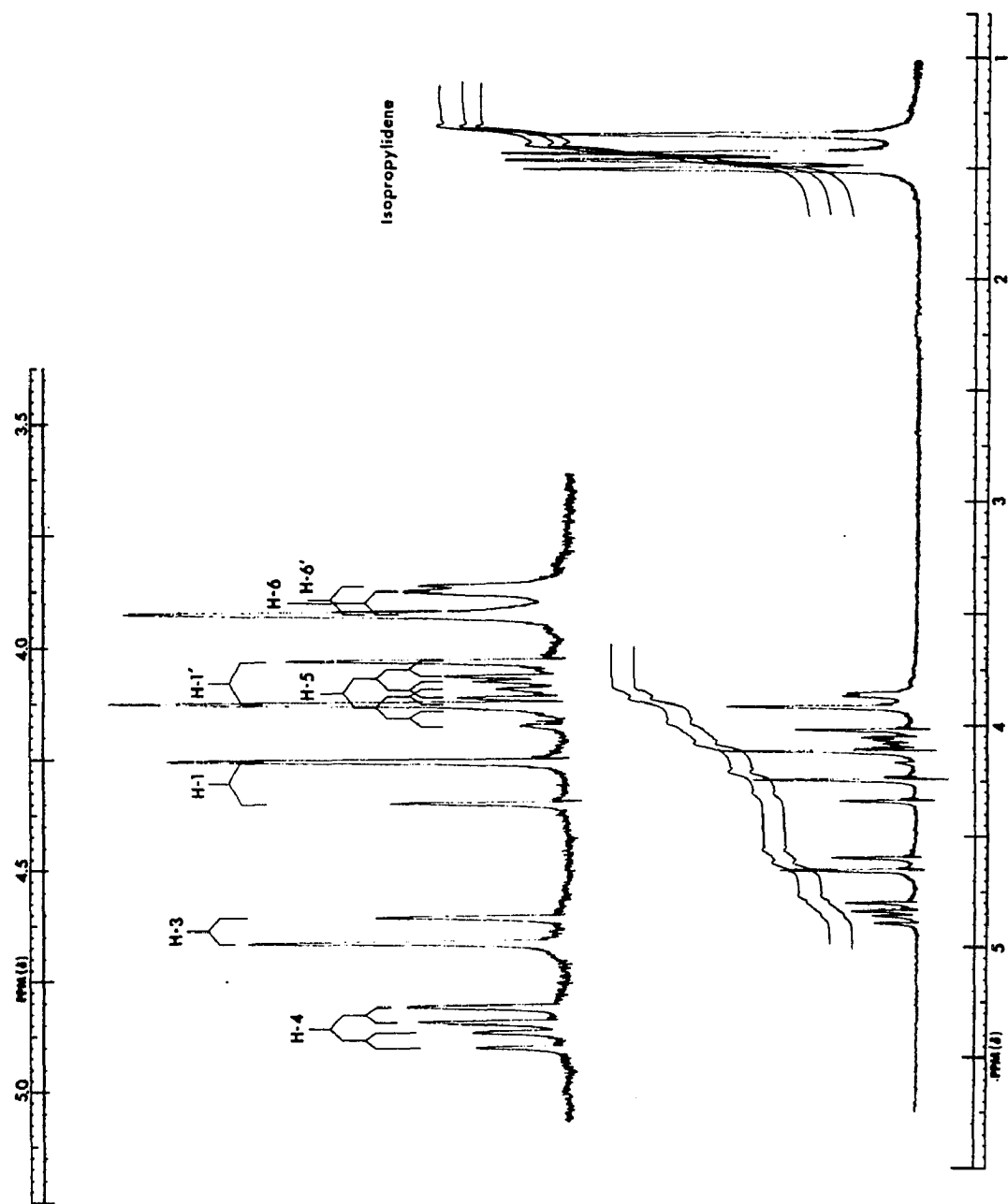


Fig. IV-9. Proton NMR spectrum of 1,2:3,4-di-O-iso-propylidene- α -D-tagatofuranose (XI) recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton.



Elemental analysis — for formula $C_{12}H_{20}O_6$ (260.29),

Calculated: C 55.37, H 7.75

Found: C 55.45, H 7.72

5. Synthesis of L-Sorbose 6-Phosphate IV)

2,3-O-Isopropylidene- α -L-sorbofuranose 1,6-Bis-(diphenylphosphate XII) — 2,3:4,6-Di-O-isopropylidene- α -L-sorbofuranose was selectively hydrolyzed (19, 20) and phosphorylated (11) to form XII, which was homogeneous by thin layer chromatography (R_F 0.36, diethyl ether) after recrystallization from ethanol-water; m.p. 100.5-101.5°, $[\alpha]_D^{23} - 3.85 \pm 0.06$ (5.0 g per 100 ml of chloroform); values reported in the literature (11): m.p. 100-101°, $[\alpha]_D^{23} - 4.2^\circ$. The proton NMR of this intermediate at 60 MHz in deuteriochloroform was consistent with its assigned structure. Chemical shifts with respect to tetramethylsilane are, 1.20 δ and 1.40 (two 3-proton singlets, isopropylidene methyls), 4.12 (1-proton multiplet, hydroxyl), 4.26-4.70 (7-proton multiplet, H-1, H-1', H-3, H-4, H-5, H-6, H-6') and 4.21 (20-proton multiplet, 4 phenyls).

L-Sorbose 6-P (IV) — Using the procedure of Mann and Lardy (11) XII was subjected to catalytic hydrolysis to eliminate the isopropylidene residue and the labile (C-1) phosphate. The resulting L-sorbose 6-phosphate (IV) was isolated as its hydrated barium salt. It was found pure by

elemental analysis; $[\alpha]_D^{29} = 11.7 \pm 0.3^\circ$ (2.0 g per 100 ml of water), literature value (11) $[\alpha]_D^{29} = 12$. This barium salt gave the following IR data (λ_{\max}): 3.00 μ (OH), 3.45 (CH), 5.85, 6.15 (OH), 6.30 & 6.75 (CH), 8.15, 9.00 (COC), 9.25 (P=O), 10.27 (POC) 11.30, and 13.10 (POC).

6. Assay of Phosphofructokinase Activity

Procedures and conditions for the assay of rabbit muscle phosphofructokinase were identical to those described in "Materials and Methods" (Section B), Part 5 of Chapter III.

C. Results

1. Synthesis of D-Psicose 6-Phosphate (II, Fig. IV-2)

1,2:3,4-Di-O-isopropylidene- β -D-psicofuranose 6-Di-phenylphosphate (X) — At 0° , 1.2 ml (5.6 mmoles) of di-phenyl phosphochloridate was added to a well-stirred solution of 1.0 g (3.8 mmoles) of 1,2:3,4-di-O-isopropylidene- β -D-psicofuranose (IX) in 10 ml of dry pyridine. Stirring was continued for 20 more min. at 0° and the reaction mixture was then left to stand for 48 hr. at 4° . The excess phosphorylating agent was then destroyed by addition of 2 ml of water. After standing at room temperature for 45 min., the reaction mixture was added slowly to 150 ml of stirred ice water. The resulting emulsion was stirred for 45 min., then transferred to a separatory funnel, and extracted twice with 100 ml of chloroform. The extraction

was facilitated by addition of 40 ml of saturated sodium chloride solution. The chloroform extracts were combined, washed twice with 100 ml of ice cold 1 N sulfuric acid and once with 100 ml of saturated sodium bicarbonate solution, dried over excess anhydrous sodium sulfate, and concentrated under reduced pressure. The oily product was taken up in 50 ml of absolute ethanol and water was added to turbidity. After vigorous scratching and storage at -20° for 36 hr., a white solid formed, which was recrystallized from cold aqueous ethanol yielding short white needles (1.71 g, 92%), m.p. $65-66^{\circ}$. This material was homogeneous by thin layer chromatography (R_F 0.48, Solvent 3); $[\alpha]_D^{20} - 55.1 \pm 0.5^{\circ}$ (2.0 g per 100 ml of chloroform); IR data (λ_{\max}) are: 2.95 μ (OH), 3.40 and 3.45 (CH), 6.28 and 6.75 (Ph); 6.88 (CH₂), 7.28 and 7.33 [C(CH₃)₂], 7.75 (P=O), 7.85, 7.93, 8.00, 8.20, 8.25, 8.33 and 8.38 (all form, POPh), 8.60 and 8.65 (1,3-dioxolane), 9.10, 9.33 (COC), 9.53 (1,3-dioxolane), 9.78 (P=O), 9.93, 10.43 (POPh), 10.53 (POC), 11.03, 11.33 and 11.63 (all three, furanose acetals), 12.08 [C(CH₃)₂], 12.85 (Ph), 12.98, 13.08 and 13.23 (POC), and 14.53 (Ph). The proton NMR spectrum of (X) was consistent with the assigned structure (Tables IV-1 and IV-2).

Elemental analysis — for formula $C_{24}H_{29}O_9P$ (492.46),

Calculated: C 58.51, H 5.93, P 6.28

Found: C 58.46, H 6.10, P 6.30

D-Psicose 6-Phosphate (II)— A solution of 493 mg (1.0 mmole) of 1,2:3,4-di-O-isopropylidene- β -D-psicofuranose 6-diphenylphosphate (X) in 25 ml of anhydrous methanol was reduced over platinum oxide (25 mg) in a Parr catalytic hydrogenation apparatus at 3.5 atm of pressure. After the uptake of 8.2 mmoles (100%) of hydrogen, the reaction was complete (6 hr). The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to a viscous oil which could not be induced to crystallize from hexane. The oily product was suspended in 3 ml of water and heated on a hot plate for 5 min. The resulting clear, acidic solution was made alkaline (pH 10) with saturated aqueous barium hydroxide solution. After the excess base was precipitated by gassing with carbon dioxide, the reaction mixture was filtered. The combined filtrate and water washings (4 ml) were added in a thin stream to 20 ml of absolute ethanol. The resulting precipitate was thoroughly suspended, allowed to flocculate at 4° overnight, collected by centrifugation, washed twice with absolute ethanol and once with diethyl ether, and finally dried in vacuo overnight. The yield was 313 mg (76%). As its diammonium salt, this material was chromatographically homogeneous (R_F 0.14 on paper using Solvent 2); $[\alpha]_D^{20} + 11.1 \pm 0.1^\circ$ (2.0 g of the barium salt per 100 ml of water); IR data (λ_{max} for barium salt) are: 3.00 μ (OH), 3.45 (CH), 5.80, 6.15 (OH), 6.95 and 7.20 (CH), 8.00, 9.00 (COC),

9.25 (P=O), 10.25 (POC), 10.80, and 12.65 (POC).

Elemental analysis—for formula $C_6H_{11}O_9P\text{Ba} \cdot 4 H_2O$ (467.52)

Calculated: C 15.41, H 4.10, P 6.63

Found: C 15.33, H 4.16, P 6.69

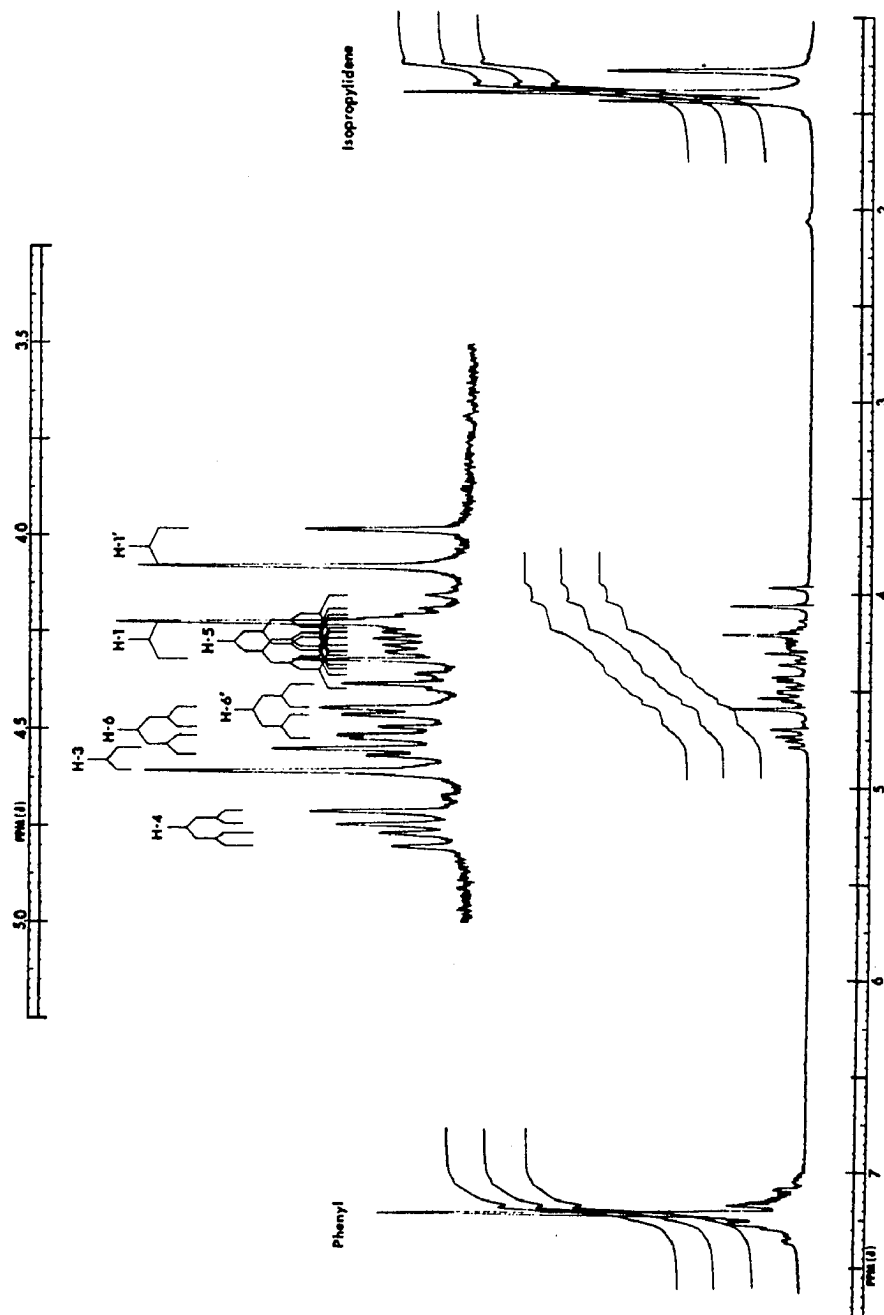
2. Improved Procedure for the Synthesis of D-Tagatose 6-Phosphate (III)

1,2:3,4-Di-O-isopropylidene- α -D-tagatofuranose 6-Diphenylphosphate (XIII)—To a well-stirred solution of 500 mg (1.90 mmoles) of desiccated 1,2:3,4-di-O-iso-propylidene- α -D-tagatofuranose in 5 ml of dry pyridine at 0° was added 0.60 ml (2.80 mmoles) of diphenyl phosphochloridate. After the mixture was stirred for 30 min. at 0° and allowed to stand 60 hr. at 4°, excess phosphorylating reagent was destroyed by addition of 1 ml of water and the stirring was continued for 1 hr. The reaction mixture was then processed in a manner identical to that described above for the psicofuranose derivative (X). The product was a clear yellow oil which was taken up in 25 ml of ethanol and water was added to turbidity (approx. 25 ml). Upon storage at 4° for 22 hr., a white solid formed, which was recrystallized from cold aqueous ethanol yielding white needles (861 mg. 93% yield); m.p. 94-95°, $[\alpha]_D^{20} + 32.3 \pm 0.5^\circ$ (2 g per 100 ml of chloroform). Literature values (10) are: yield 86%, m.p. 94-95°, $[\alpha]_D^{20} + 32^\circ$. The final

product was homogeneous by thin layer chromatography (R_F 0.41, Solvent 3). IR data (λ_{\max}) are: 2.95 μ (OH), 3.30, 3.40 and 3.45 (all three, CH), 6.30 and 6.75 (Ph), 6.90 (CH_2), 7.28 and 7.33 [$\text{C}(\text{CH}_3)_2$], 7.80 ($\text{P}=\text{O}$), 7.95, 8.25 and 8.43 (both POPh), 8.58 and 8.63 (1,3-dioxolane), 8.83, 9.00, 9.25 (1,3-dioxolane), 9.35 (COC), 9.65 ($\text{P}=\text{O}$), 9.93, 10.40 (POPh), 10.55 (POC), 10.93, 11.45, 11.63 and 11.83 (all four, furanose acetals), 12.23 [$\text{C}(\text{CH}_3)_2$], 12.85 (Ph), 12.98, 13.05 and 13.25 (all three, POC), and 14.53 (Ph). The proton NMR spectrum of XIII was consistent with the assigned structure (Tables IV-1 and IV-2 and Fig. IV-10).

1,2:3,4-Di-O-isopropylidene- α -D-tagatofuranose 6-Phosphoric Acid (XIV)—A solution of 260 mg (0.528 mmoles) of 1,2:3,4-di-O-isopropylidene- α -D-tagatofuranose 6-diphenylphosphate in 25 ml of anhydrous methanol was reduced over platinum oxide (25 mg) at 2 atmospheres of pressure. The reaction was complete after the uptake of 4.4 mmoles (112%) of hydrogen (4.3 hr). The platinum catalyst was removed by filtration and the filtrate was concentrated in vacuo to a viscous acidic oil, which was triturated with 20 ml of hexane and stored at 4° overnight. The crystalline material that formed was filtered, washed with hexane, and dried in vacuo; yield 166 mg (93%) of white needles, m.p. 113-114° (with decomposition), $[\alpha]_D^{25} + 47.0 \pm 0.5^\circ$ (2 g per 100 ml of absolute methanol). Literature values (10) are: yield 33.4%, m.p. 92°, $[\alpha]_D^{25} + 43.6^\circ$.

Fig. IV-10. Proton NMR spectrum of 1,2:3,4-di-O-iso-propylidene- α -D-tagatofuranose 6-diphenylphosphate (XIII) recorded at 100 MHz in chloroform-d. The upper trace is an expansion of signals from the methylene and methine protons attached to the sugar skeleton.



D-Tagatose 6-Phosphate (III)—A suspension of 150 mg (0.441 mmoles) of 1,2:3,4-di-O-isopropylidene- α -D-tagatofuranose 6-phosphoric acid and 1.0 ml of water was heated on a hot plate for 4 min. with stirring. The resulting acidic solution was neutralized and the barium salt of D-tagatose 6-P was precipitated using the same procedure described above for the psicofuranose analogue (II). The product weighed 164 mg (94% yield, overall 81%) and was pure by elemental microanalysis and paper chromatography (R_F 0.15, Solvent 2), $[\alpha]_D^{25} + 6.0 \pm 0.4^\circ$ (1.06 g of barium salt per 100 ml of water). Literature values (10) are: 75% yield, overall 64%; $[\alpha]_D^{25} + 5.65^\circ$. IR data (λ_{\max}): 3.00 μ (OH), 3.45 (CH), 5.85, 6.15 (OH), 7.15 (CH), 7.48, 8.00, 9.00 (COC), 9.25 (P=O), 10.25 (POC), 10.80, 11.15, and 12.55 (POC).

3. Phosphofructokinase Activity of Analogues

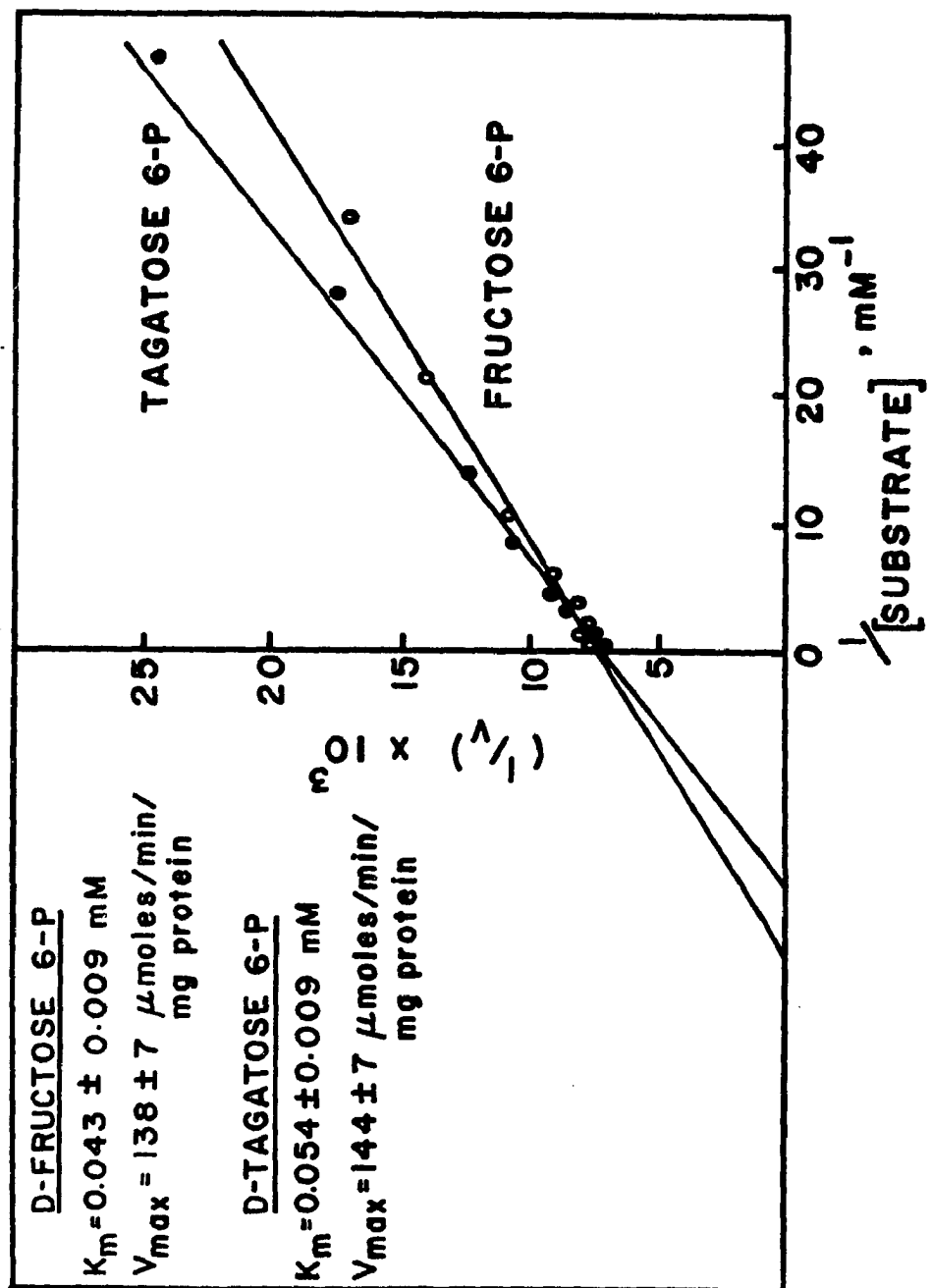
In our hands, all three epimeric analogues of D-fructose 6-P exhibited an activity as alternate substrates for rabbit muscle phosphofructokinase, although to different extents. Table IV-3 summarizes the kinetic constants of these analogues. D-Tagatose 6-P (III), the C-4 epimer of D-fructose 6-P (I), was found to be an excellent substrate for phosphofructokinase. Within experimental error, the kinetic constants of this analogue were identical to those of the natural substrate (Fig. IV-11). Although D-tagatose 6-P has been known to be an alternate substrate for

TABLE IV-3

Comparison of the kinetic constants of substrates of rabbit muscle phosphofructokinase at pH 7.8, 28° and 1 mM ATP. Other conditions are as described under "MATERIALS AND METHODS" (Section B), Part 5 of Chapter III.

| Substrate and epimeric carbon | K_m , mM | $\log \frac{K_{m,epimer}}{K_{m,F6P}}$ | Relative V_{max} |
|----------------------------------|-------------|---------------------------------------|--------------------|
| <u>D</u> -Fructose 6-P | 0.043±0.009 | 0 | 100 |
| <u>D</u> -Psicose 6-P, C-3 | 3.0±0.6 | 1.84 | 45 |
| <u>D</u> -Tagatose 6-P, C-4 | 0.054±0.009 | 0.10 | 104 |
| <u>L</u> -Sorbose 6-P, C-5 | 11±2 | 2.41 | 15 |

Fig. IV-11. Lineweaver-Burk plot of the initial rates of phosphorylation of D-fructose 6-P and D-tagatose 6-P as a function of substrate concentration. Assay conditions are as described under "MATERIALS AND METHODS." Data courtesy Mr. A. E. Ashour (21).



phosphofructokinase (12,13), this is the first quantitative measurement of the latter's high affinity for this sugar phosphate.

D-Psicose 6-P (II), and L-sorbose 6-P (IV), the C-3 and C-5 epimers, respectively, were found to act as very poor substrates. Their K_m 's were approximately two orders of magnitude higher than the K_m of fructose 6-P (Table IV-3). Our finding that L-sorbose 6-P has a very low, but measurable, activity contradicts a recent report (6) that this analogue is inactive.

D. Discussion

1. Interpretations of the Effects of Epimerization

In a previous communication (3), we provided evidence that phosphofructokinase acts only on the β -anomer of D-fructofuranose 6-P. Using ^{13}C NMR spectroscopy, we (1) have also shown that this anomer is the predominant form in aqueous solutions of D-fructose 6-P. In the light of these findings, the failure of D-psicose 6-P and L-sorbose 6-P to act as good substrates could be attributed to a primary and/or a secondary effect. The primary effect would be due to alteration in the bond formed directly between the epimeric hydroxyl and a functional residue on the enzyme. The secondary effect would be an anomeric one, i.e., the change of configuration of C-3, C-4, or C-5 might induce a change in the anomeric composition of the substrate in

solution. These two possibilities are discussed further below.

2. Epimeric Specificity (Configurational Specificity at C-3, C-4, and C-5)

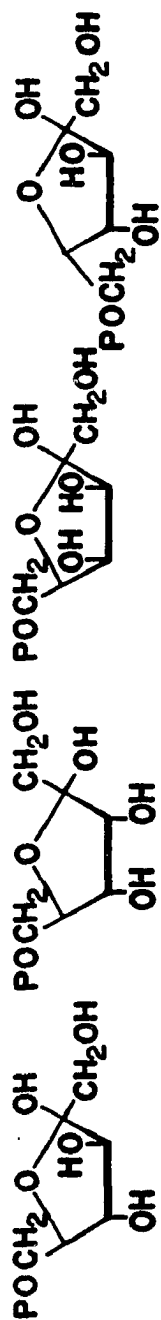
The following conclusions can be drawn from a comparison of the kinetic constants of the analogues tested with those of D-fructose 6-P (Table IV-3):

(1) The L-configuration at C-3 of the furanose substrate is necessary for efficient enzymatic activity.

In all probability, the importance of the C-3 configuration is due primarily to the formation of a hydrogen bond between the C-3 hydroxyl group of the substrate and a precisely oriented functional group at the active site of the enzyme.

On the other hand, the secondary effect referred to above could be invoked here as a contributing factor to the 100-fold loss in affinity upon the epimerization of the configuration around C-3. Recently, Que and Gray (22) have shown that ketohexofuranose sugars exist predominantly in solution such that the C-1 hydroxymethyl group and C-3 hydroxyl group are disposed in a transoid relationship. Accordingly, D-psicofuranose 6-P should exist predominantly as its α -anomer in solution (II α , Fig. IV-12). This conclusion has been supported experimentally in our laboratory (1). However, we believe that this secondary

Fig. IV-12. The predominant anomeric form of D-fructofuranose 6-P and its epimers in solution (1,7). To be noted is the equivalence of the α -L and the β -D configurations.



| | | | |
|--|--|--|--|
| β-D-FRUCTO- FURANOSE 6-P | α-D-PSICO- FURANOSE 6-P | β-D-TAGATO- FURANOSE 6-P | α-L-SORBO- FURANOSE 6-P |
| I β | II α | III β | IV α |

effect cannot explain the drastic drop in affinity since the rate of spontaneous anomerization between the α - and β -forms of the ketohexofuranose 6-P substrates is probably much more rapid than the enzymatic rates of phosphorylation of any of these substrates, due to the high concentrations of substrates and limited amounts of phosphofructokinase present in our assay mixture.

The importance of the configuration around C-3 is further supported by the affinity of phosphofructokinase toward the two epimeric pentoses: D-ribose 5-P and D-arabinose 5-P. The presence of the L-configuration at the C-2 of D-arabinose 5-P (comparable to the C-3 of D-fructose 6-P) results in an effective inhibitor for this enzyme with a $K_i = 1.0$ mM (3,4). A change to the D-configuration around C-2 renders the resulting compound, D-ribose 5-P, inactive (13).¹

(2) The configuration around C-3 is of no detectable importance in enzymatic activity. The nearly identical kinetic constants for D-fructose 6-P (I) and D-tagatose 6-P (III) lead to this conclusion. This case is uncomplicated by consideration of a secondary effect since a configurational change at C-4 has no effect on the anomeric composition of the furanose substrate (7) in solution.

¹ T.A.W. Koerner, Jr., R.J. Voll and E.S. Younathan, unpublished data.

(3) The D-configuration around C-5 is necessary for efficient enzymatic activity. The data in Table I indicate that L-sorbose 6-P (IV) acts as a very poor substrate for phosphofructokinase. The inversion of the configuration at C-5 leads to a several Angstrom displacement of the C-6 hydroxyl of the substrate (Fig. IV-12). Since the C-6 hydroxyl bears the phosphoryl group, C-5 epimerization leads to improper positioning of what is probably the most important binding group of the substrate. No secondary effect can be invoked in this case, since C-5 epimerization should have no effect on the anomeric composition of the furanose substrate (22) in solution.

It is interesting to note that the K_m of phosphofructokinase for L-sorbose 6-P is comparable to its K_m for D-fructose 6-sulfate (23) and D-tagatose 6-sulfate.¹ Since the configuration at C-5 in the latter two analogues is identical to that in fructose 6-P, the 100-fold increase in K_m can be attributed primarily to the loss of one negative charge upon the substitution of the phosphate with a sulfate group. Consequently, one can presume that the inversion in the configuration at C-5 still allows the binding of L-sorbose 6-P to the enzyme, but through only one of the two electronegative charges of the phosphate group.

3. Physiological Significance of the Epimeric Specificity of Phosphofructokinase

The specificity of phosphofructokinase for the L-configuration at C-3 is consistent with that of other enzymes of D-fructose metabolism, namely yeast (24-26) and brain (27) hexokinase, liver fructokinase (24), and muscle aldolase (28,29). Thus, it seems that these related enzymes have evolved with identical specificity for this part of the substrate molecule. This conservation of structural requirement is consistent with maximal metabolic efficiency and is of obvious advantage in natural selection (30) at the molecular level.

The lack of specificity at C-4 is not readily evident. However, it is conceivable that certain biological systems are capable of phosphorylating D-tagatose to its 1,6-diphosphate. Reports in the literature indicate that D-tagatose exists in plants (31,32) and that both fructokinase (24) and aldolase (28) act on D-tagatose and D-tagatose 1,6- P_2 , respectively. These observations suggest that the ability of phosphofructokinase to act efficiently on D-tagatose 6-P may have physiological significance as part of a D-tagatose metabolizing pathway.

Such a pathway might play a role in galactosemia. In this pathological state, the Leloir uridine nucleotide pathway (33) is congenitally non-functional, yet small amounts of galactose are metabolized (34-36). In this case, the following pathway might be operative: D-

galactose 6-P \rightarrow D-tagatose 6-P \rightarrow D-tagatose 1,6-P₂ \rightarrow D-glyceraldehyde 3-P plus dihydroxyacetone-P. Such a "D-tagatose 6-P pathway" has been demonstrated in Staphylococcus aureus (37,38) and in group N streptococci (39). However, its existence in mammalian systems remains to be studied. Another possible metabolic route would be the "sorbitol pathway": D-galactose \rightarrow galactitol \rightarrow D-tagatose. Subsequently, D-tagatose would be acted upon by fructokinase to give D-tagatose 6-P. This sorbitol pathway has been shown to exist in certain mammalian tissues (40), although the available data suggest that its rate of galactitol utilization is lower than that of other hexitols.

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APPENDIX
TABLE OF ABBREVIATIONS

| | |
|---------------------|--|
| Ac | Acetyl |
| AM1P | 2,5-Anhydro- <u>D</u> -mannitol 1-phosphate |
| AG6P | 2,5-Anhydro- <u>D</u> -glucitol 6-phosphate |
| atm. | Atmosphere |
| ATP | Adenosine 5'-triphosphate |
| Bz | Benzoyl |
| ^{13}C | Carbon-13, isotope of carbon |
| CMR | Carbon-13 magnetic resonance |
| DBF | Dibenzylidene <u>D</u> -fructose |
| DCCl ₃ | <u>Deuterio</u> -chloroform |
| DMSO | Dimethyl sulfoxide |
| DMSO-d ₆ | Hexa- <u>deuterio</u> -dimethylsulfoxide |
| FDP | <u>D</u> -Fructose 1,6-diphosphate |
| FF | <u>D</u> -Fructofuranose |
| F6P | <u>D</u> -Fructose 6-phosphate |
| g | Gram |
| IR | Infrared |
| K _m | Michaelis constant (reciprocal measure of enzyme-substrate affinity) |
| μg | Microgram |
| m.p. | Melting point (corrected) |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide (reduced) |

APPENDIX (cont'd)

| | |
|------------------|---|
| NMR | Nuclear magnetic resonance |
| PEP | Phosphoenol pyruvate |
| Ph | Phenyl |
| pyr | Pyridine |
| TLC | Thin layer chromatography |
| TMS | Tetramethyl silane |
| Tris Cl | <u>Tris</u> -(hydroxymethyl)-aminomethane hydrochloride |
| UV | Ultraviolet |
| v | Velocity |
| V _{max} | Maximal velocity (of an enzyme reaction) |

VITA

Theodore Alfred William Koerner, Jr., was born in Waco, Texas, on July 30, 1947, the first child of Isabella Yerkes Koerner and Dr. T. A. W. Koerner, Sr., a physician in general medical practice. Prior to their marriage both the author's parents had pursued careers in science and medicine. The author's mother had been a bacteriologist at Walter Reed Hospital and his father a research bacteriologist at the University of Wisconsin (M.A., 1939) and, during medical school, at the University of Pennsylvania (M.D., 1943).

The author first attended public school in McKinney, Texas, where his father was a resident in pathology, then in Shreveport, Louisiana, where his father had accepted a position as Chief of Laboratory Services and Pathologist at the Veterans Administration Hospital. Several Shreveport public school teachers stand out in the author's mind as having further encouraged an interest in science that had begun at home. These are Mrs. M. D. Schaal, Mrs. N. Brewster, Mrs. D. Hubble, and Dr. G. Pennington. The author graduated from C. E. Byrd High School in 1965.

After attending Centenary College of Shreveport for one year and the University of Texas for one year, the author decided on biochemistry as a major and matriculated in the fall of 1967 at Louisiana State University in Baton Rouge. The interdisciplinary nature of this curriculum was

stimulating, allowing the author to experience the pristine beauty of mathematics, the analytical power of physical chemistry, and the incomparable challenge of organic synthesis. Always the target of these studies was the elucidation of the fascinating logic of living state. Having found his life's work, the author received the B.S. degree in biochemistry in 1970.

As an undergraduate in 1968 the author had met Dr. E. S. Younathan and begun research in his laboratory in the summer of 1969. Thus in 1970 the author began graduate studies in biochemistry at LSU under the supervision of Dr. Younathan. The fruitful research begun in Dr. Younathan's laboratory has continued unabated up to the present and is the basis for the author's candidacy for the degree of Doctor of Philosophy.

In 1971 the author married the former Janice Rose Shipp, daughter of Dr. and Mrs. James H. Shipp of Shreveport, Louisiana.

In 1973 the author followed the advice of Dr. Younathan, the tradition of his family, and the dictum of Otto Warburg* and applied for admission to Medical School. The author is presently a sophomore medical student at Tulane Medical School in New Orleans, Louisiana. It is

* "A biochemist should not be afraid to tackle the great problems of biology."

his hope to pursue a career in medical research.

The author is a member of the following honorary societies: Phi Lambda Upsilon (chemical, 1973), Alpha Epsilon Delta (pre-medical, 1973), and Sigma Xi (research, 1975, full membership).

The author has presented papers at four scientific gatherings. These were the meetings of the Southwestern Region of the American Chemical Society in New Orleans (1970) and Baton Rouge (1972), and the American Society of Biological Chemists in Atlantic City (1973) and Minneapolis (1974).

The author's research has resulted in the following publications:

1. Koerner, T. A. W., Jr., Younathan, E. S. and Wander, J. D. "Elucidation of the Structure of Dibenzylidene Fructose by Physical Methods and Use of a Shift Reagent" (1972) Carbohyd. Res. 21, 455.
2. Koerner, T. A. W., Jr., Cary, L. W., Bhacca, N. S. and Younathan, E. S. "Tautomeric Composition of D-Fructose Phosphates in Solution by Fourier Transform Carbon-13 Nuclear Magnetic Resonance" (1973) Biochem. Biophys. Res. Commun. 51, 543.
3. Koerner, T. A. W., Jr., Ashour, A. E., Voll, R. J. and Younathan, E. S. "The Anomeric Specificity of Phosphofructokinase" (1973) Federation Proceedings 32, 668.
4. Koerner, T. A. W., Jr., Ashour, A. E., Voll, R. J. and Younathan, E. S. "The Phosphoryl Acceptor Site of Phosphofructokinase. Substrate Specificity and a Model for Binding" (1974) Federation Proceedings 33, 499.

5. Koerner, T. A. W., Jr., Younathan, E. S., Ashour, A. E. and Voll, R. J. "The Fructose 6-Phosphate Site of Phosphofructokinase. I. Tautomeric and Anomeric Specificity" (1974) J. Biol. Chem. 249 5749.
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EXAMINATION AND THESIS REPORT

Candidate: Theodore Alfred William Koerner, Jr.

Major Field: Biochemistry

Title of Thesis: FRUCTOSE PHOSPHATES AND THEIR STRUCTURAL ANALOGUES. Synthesis, Nuclear Magnetic Resonance Studies, and Use in the Exploration of the Active-Site of Phosphofructokinase

Approved:

Ezra S. Lownathan
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Date of Examination:

December 2, 1975